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Development and applications in affinity chromatography
and enzyme technology

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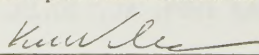
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SÖLVEGATAN 39, HÖRSAL A, ONSDAGEN DEN 9 MAJ 1984, KL. 10.15.

Organization LUND UNIVERSITY Department of Pure and Applied Biochemistry University of Lund, Chemical Center, POB 740, S-220 07 Lund, Sweden		Document name DOCTORAL DISSERTATION	
		Date of issue April, 1984	
		CODEN: LUTKDH/(TKBK-1009)/1-160/1984	
Author(s) Kurt Nilsson		Sponsoring organization	
Title and subtitle A NEW IMMOBILIZATION METHOD BASED ON ORGANIC SULFONYL HALIDES Development and applications in affinity chromatography and enzyme technology			
Abstract An immobilization method suitable for activation of hydroxyl group containing supports, i. e. agarose, cellulose, glycophasse-glass etc., is described. The support is treated with an organic sulfonyl chloride, e. g. p-toluenesulfonyl chloride (tosyl chloride) or 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride), with the rapid formation of the corresponding sulfonate esters. Water-soluble polymers, such as poly(ethylene glycol), are also easily derivatized with this technique. Supports activated with tresyl chloride were found to be suitable for coupling of pH-sensitive enzymes and affinity ligands with high yields even at neutral pH. Conjugates of agarose or glycerylpropyl-silica containing 300 mg soybean trypsin inhibitor per g of dry support were thus prepared. Immobilization generally resulted in high retention of enzymatic activity. Thus, horse liver alcohol dehydrogenase (HLADH) was coupled in almost 100 % yield with virtually complete retention of specific activity and coenzyme binding properties. Immobilized affinity ligands, e. g. N ⁶ -(6-aminohexyl)-5'-AMP, soybean trypsin inhibitor, concanavalin A and protein A, were shown to have retained their affinity properties, as demonstrated by affinity chromatography. In the pH-range 9 to 10.5 tosylated supports are effective for coupling of ligands that are stable in this range. Efficient coupling of ligands not soluble in water can be obtained using organic solvent. The possibility of utilizing an enzyme, HLADH, immobilized to tresyl-silica in an HPLC system for affinity separations on an analytical scale, together with its use in the screening of K _d for retained material, was demonstrated. Peptide synthesis in aqueous-organic solvent mixtures were studied using chymotrypsin immobilized to tresyl-agarose. With the kinetic (non-equilibrium) method, the yield of peptide was found to be strongly dependent on temperature and on concentration and type of organic solvent. The stability of the immobilized enzyme was influenced by the amount of tresyl chloride used during activation of agarose.			
Key words immobilization, agarose, cellulose, glass, poly(ethylene glycol), tosyl chloride, 2,2,2-trifluoroethanesulfonyl chloride, enzyme, affinity ligand, affinity chromatography, HPLC, peptide synthesis			
Classification system and/or index terms (if any)			
Supplementary bibliographical information			Language English
ISSN and key title			ISBN
Recipient's notes		Number of pages 160	Price
Security classification			

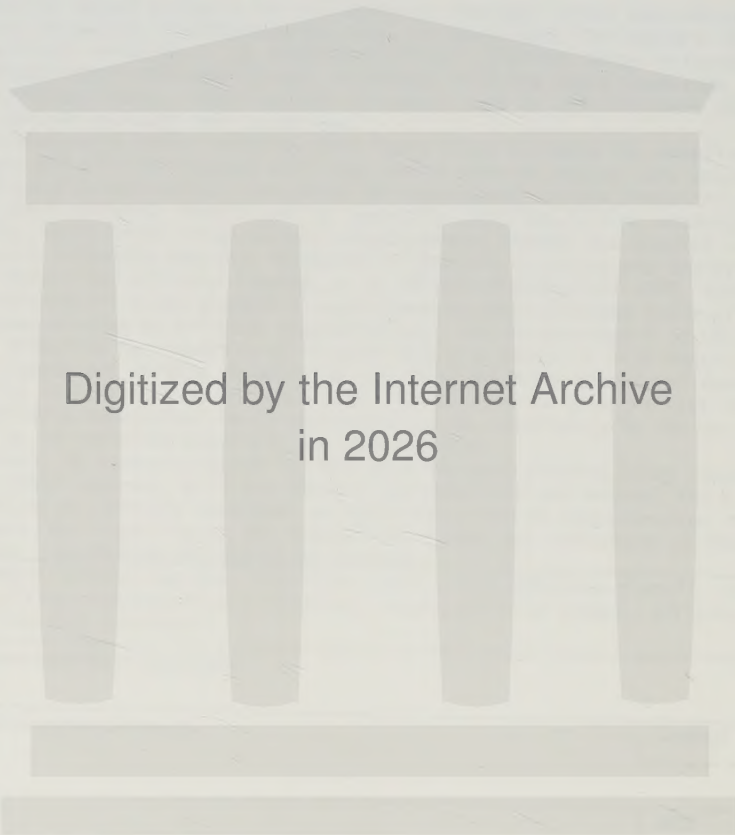
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Date April 9, 1984

To my parents,
Pia and Jenny



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This thesis is based on the following papers, which are referred to by Roman numerals in the text.

- I K. Nilsson and K. Mosbach: p-Toluenesulfonyl chloride as an activating agent of agarose for the preparation of immobilized affinity ligands and proteins.
Eur. J. Biochem. 112, 397-402 (1980).
- II K. Nilsson, O. Norrlöw and K. Mosbach: p-Toluenesulfonyl chloride as an activating agent of agarose for the preparation of immobilized affinity ligands and proteins. Optimization of conditions for activation and coupling.
Acta Chem. Scand. B35, 19-27 (1981).
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Biochem. Biophys. Res. Commun. 102, 449-457 (1981).
- IV K. Nilsson and K. Mosbach: Peptide synthesis in aqueous-organic solvent mixtures with α -chymotrypsin immobilized to tresyl chloride activated agarose.
Submitted for publication
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Anal. Biochem. 134, 60-72 (1983).



I INTRODUCTION

During the past millenia mankind has utilized enzymes for production of food and beverages, in the textile industry, for the preparation of hides and in the medicinal industries. Today, with over 2000 enzymes isolated and at least partially characterized, research on their application is increasing. Enzyme technology could offer many advantages compared with conventional chemical technology. For example, the amounts of side products are expected to be reduced, and asymmetric synthesis can be achieved. When considering the need for technologies characterized by low energy consumption and minimal waste production, the interest in enzyme technology becomes even more evident.

The application of enzymes as catalysts are however hampered by their often relatively low stability and high cost. Methods that facilitate reutilization and stabilization of the enzyme is therefore desirable.

By immobilizing the enzyme (i.e. by physically confining or localizing the enzyme in a region of space with retention of its activity during a continuous process) this can often be achieved. In addition, the enzyme can be easily removed from the solution without contamination of the reaction mixture. Immobilization techniques aimed at achieving those advantages have been the subject of increased interest since the end of the 1960s (1-3).

In addition to facilitate the use of enzymes for syntheses of useful compounds, the immobilization also makes possible to arrange specific and sensitive enzymes in proximity to a transducer, making continuous analysis of complicated technical and clinical reaction mixtures possible. Examples of such devices are the enzyme electrode (4) and the enzymes thermistor (2,5).

Abbreviations:

HLADH = horse liver alcohol dehydrogenase; STI = soybean trypsin inhibitor; HPLAC = high performance liquid affinity chromatography; tresyl chloride = 2,2,2-trifluoroethanesulfonyl chloride; Ac-Phe-OMe = N-acetyl-phenylalanine methyl ester; Ac-Phe-OEt = N-acetyl-phenylalanine ethyl ester; H-Gly-NH₂ = glycynamide; H-Ala-NH₂ = alaninamide. The constituent amino acids were of the L-configuration unless otherwise stated.

Immobilized enzymes have also been thought to have therapeutical potential for removal of toxic substances from body fluids and replacement of missing or defective enzymes. As an example, immobilized albumin in an extracorporeal shunt system has been utilized for the removal of bilirubin released in cases of liver injury (6). This area is however still in its infancy.

Furthermore, immobilized enzymes and immobilized multienzyme systems can provide models of conditions in vivo, since an increasing number of enzymes are thought to be immobilized in the cell. Thus, several of the glycolytic enzymes bind tightly to muscle protein and a number of enzymes acting in sequence are immobilized to the mitochondrial and cellular membrane. Immobilization per se has been used as a tool to study enzyme mechanism (7,8), subunit interactions in multimeric enzymes (9,10), refolding of enzymes (11) and the behavior of enzymes in aqueous-organic solvent mixtures (12). Studies aimed at altering the properties of enzymes by immobilization have also been undertaken. Examples are coimmobilization of phosphorylase b with its effector AMP, yielding a preparation that does not require free AMP for optimal activity (13) and attempts to immobilize trypsin in its substrate binding conformation (14).

The principle of immobilization has also been taken advantage of for purification of enzymes. Affinity chromatography, which exploits the biospecific, reversible interaction between a macromolecule (usually an enzyme) and its immobilized ligand (substrate, inhibitor, effector, etc) has been extensively employed for the selective purification of macromolecules (15-18). A prerequisite for the successful development of this technique has been the introduction of efficient immobilization methods allowing covalent attachment of affinity ligands to solid supports. The development of the two fields, affinity chromatography and immobilized enzymes, has been closely interrelated since the same procedures for covalent immobilization of affinity ligands and enzymes to solid supports can be used.

Some of the reactions utilized for immobilization have also been useful for the covalent binding of affinity ligands to watersoluble polymers, such as poly(ethylene glycol) and dextran (19). These polymer ligand derivatives have been applied in aqueous polymer two-phase systems for purification of enzymes (affinity partitioning) (20).

As enzymes are quite costly and fragile, much work is currently being put into the modelling of their action by cheaper, more stable compounds (21). Immobilization might be an aid both for the construction of such models and for their reutilization. Although still in an embryonic state, steps in this direction have been taken (22,23).

This thesis is devoted to the development of new immobilization procedures for both enzymes as well as affinity ligands. Methods for covalent immobilization of enzymes and affinity ligands to hydroxyl group carrying supports, using organic sulfonyl chlorides are described (paper I-III) as well as the application of the preparations (paper IV and V).

In paper IV the method was used for immobilization of α -chymotrypsin to agarose. The stability of the immobilized enzyme for synthesis of dipeptides in aqueous-organic solvent mixtures was investigated. The influence of a number of parameters on peptide yield and stability was also studied.

In paper V the method was used to immobilize horse liver alcohol dehydrogenase (HLADH) to glycerylpropyl-silica. The immobilized enzyme was characterized by activity and equilibrium measurements. Integrated with HPLC-equipment it was used for rapid separations of compounds with affinity for the enzyme's coenzyme binding site. In addition, the utility of this type of system for quantitative affinity chromatography, i.e. the determination of dissociation constants from chromatographic data, was demonstrated.

II IMMOBILIZATION PROCEDURES A survey

Historically, the earliest reports on immobilization involved adsorption of proteins to a number of materials such as charcoal (24) and starch (25). In the 1930s adsorbed antigens were used for the purification of antibodies, and in 1951 the covalent binding of ovalbumin to diazotized p-aminobenzylcellulose and use of the adduct for purification of ovalbumin antibodies was reported (26). In 1953 diazotized polyaminostyrene was utilized for the first covalent immobilization of enzymes (27). Many reports dealing with new techniques for immobilization as well as the physical and chemical properties of the immobilized enzymes appeared in the fifties. However, the yield of bound protein and retained enzyme activity were generally low, presumably owing to the hydrophobicity of the supports. Entrapment of enzymes in polyacrylamide in 1963 (28), covalent immobilization to CNBr activated polysaccharides in 1967 (29), and to derivatized polyacrylamide in 1969 (30), were procedures that allowed efficient immobilization to hydrophilic supports with high retention of enzyme activity. Since then a number of various techniques for immobilization have been introduced (1-3). One of the reasons for the continued research on finding new immobilization procedures is that no one method probably can be found that is perfect for all biomolecules or purposes. Instead, the more methods that are known the better the chances of finding a method tailor-made for a specific purpose.

The methods available for immobilization of enzymes are usually classified according to the grouping introduced by Zaborsky (1):

Physical methods: Adsorption on water-insoluble support

Entrapment within water-insoluble polymer lattice

Entrapment within semipermeable membrane

Chemical methods: Covalent attachment to water-insoluble support

Crosslinking

"Hybrid methods" are also used, e.g. entrapment and covalent attachment. Physical methods rely on noncovalent interactions, while chemical methods involve at least one covalent bond formed with the biomolecule during its immobilization. For immobilization of low molecular weight affinity ligands, covalent attachment methods are generally used.

1. Adsorption

This approach is characterized by: simplicity in the immobilization step, often very short times for efficient immobilization, the wide variety of adsorbents used and, ideally, the reusability of the adsorbent. Immobilization is effected by simply mixing the enzyme solution with the adsorbent under conditions optimal for adsorption for some period of time and subsequent washing off the unbound material. The time required for immobilization mainly depends on the time for the enzyme to diffuse to the support surface (31). For supports with large pore size, a few minutes have been reported to be sufficient for optimal yield (32).

Depending on the nature of the adsorbent, the enzyme binding may be the result of hydrogen bonds and ionic, hydrophobic and/or van der Waals interactions. Biospecific interactions have also been utilized. Thus, Concanavalin A-agarose has been employed to reversibly bind glycoproteins as for example phosphodiesterase (33).

Among the supports that have been used for adsorption are ion-exchange resins (e.g. carboxymethyl- or diethylaminoethyl-substituted Sephadex or cellulose), minerals and other inorganic supports (e.g. alumina, carbon, stainless steel, glass), proteins (e.g. crosslinked collagen) and hydrophilic supports derivatized with alkylamines or hydrophobic groups (e.g. N-alkyl-agarose derivatives, phenyl-agarose) (1-3). Examples of recently developed supports are aminohexyl-cellulose coated with tannin, "immobilized tannin" (34), and trityl-agarose, i.e. agarose substituted with $(C_6H_5)_3C$ -groups (32). Immobilized tannin seems promising for continuous removal of contaminating proteins in beverages and for immobilization of enzymes (35). Trityl-agarose has been used for the successful immobilization of some labile enzymes, such as the restriction endonucleases Bam H₁ and Eco R₁ (32).

Adsorption was used in the first reported industrial application of immobilized enzymes, α -aminoacylase adsorbed onto DEAE-Sephadex was used for the continuous resolution of racemic N-acetyl-D,L-amino acids (36). The regenerability of the deteriorated immobilized enzyme columns was a main advantage with adsorption over other types of immobilization in this application.

The major difficulty with adsorption is that the conditions have to be carefully selected to minimize denaturation of enzyme activity and leakage of protein from the adsorbent. Also, the adsorbent should not adsorb reaction products or enzyme inhibitors. A charged support may cause an undesired shift in the pH-optimum of the enzyme and the enzymatic activity may be low at the pH needed for optimal adsorption (1,2). Furthermore, Michaelis constants can be considerably changed because of partitioning effects of charged or nonpolar substrates with charged or hydrophobic supports, respectively (1).

2. Entrapment

This method involved the formation of a highly crosslinked polymer network in the presence of an enzyme (1-3). The polymer lattice prevents the enzyme from diffusing into the surrounding medium, while still allowing penetration of low molecular weight substrate. It should be recognized that this type of immobilization also may involve certain adsorptive as well as covalent elements.

Gels of the polyacrylamide type are probably the most frequently used supports for entrapment. This method involves polymerization of varying amounts of acrylamide and N,N-methylene bisacrylamide in an aqueous solution containing the dissolved enzyme (2,28). For formation of small beads, a suspension polymerisation with an organic phase and an emulsifier is carried out (2, 37). Several other types of supports have been used and a process for entrapment in very fine fibers, with high surface area allowing high loading of enzyme with high retention of activity has been reported (38).

The advantages with entrapment are that the immobilization process is relatively simple and mild, and that a wide variety of gel forms can be prepared. Increased thermal stability of enzymes after entrapment have been reported. Thus, chymotrypsin entrapped in polymethacrylate showed a 10 000 fold decreased thermal denaturation rate compared with soluble enzyme or with enzyme entrapped in polyacrylamide (39). This was explained to be due to noncovalent interactions, such as hydrogen bonding and electrostatic interactions between the support and the enzyme, facilitated by largely complementary support and enzyme surfaces.

Disadvantages of entrapment include the possible inactivation of enzymes by radicals and heat liberated during polymerization, leakage of enzyme from the support and the numerous experimental factors that have to be controlled to obtain a good polymerization. In addition, substrates and products must be of low molecular weight to minimize diffusional hindrances.

To avoid the leakage problem while allowing an increased substrate permeability, entrapment has been combined with covalent attachment (2, 40, 41). In one such procedure the possible inactivation of the enzyme by the heat or radicals generated during the reaction was avoided by using a preformed, water soluble, acrylamide based polymer containing reactive N-acryloxysuccinimide, which formed an insoluble gel upon addition of enzyme and a diamine (which acted as crosslinker) instead of using monomers and radical polymerization (42).

3. Containment within semipermeable membranes

By microencapsulation, which can be regarded as a special type of entrapment, whole droplets of enzyme solution are immobilized by inclusion within microcapsules, that have either a permanent or a nonpermanent semipermeable membrane (43). Nonpermanent or liquid-surfactant membranes are prepared by mixing an aqueous enzyme solution with an organic solvent containing surfactants and additives and dispersing the so formed emulsion in an aqueous solution yielding a double emulsion (43, 44). By means of a related procedure enzymes have also been encapsulated within liposomes (45). Permanent membranes are formed either by phase separation in a polymer solution ("coacervation") or by condensation polymerisation of hydrophobic and hydrophilic monomers at the interface between organic phase and aqueous microdroplets containing the enzyme. With the condensation polymerisation procedure enzyme solutions or cell extracts can be microencapsulated within very thin, spherical membranes. This type of immobilization have been used in attempts to prepare artificial cells (46).

Related to entrapment is the containment of soluble enzymes by semipermeable membranes used in ultrafiltration cells or hollow fiber devices (1,2). These latter type of approach seems promising as an aid for the scaling up of enzyme catalyzed reactions and to facilitate reutilization of enzymes acting on high molecular weight polymeric sub-

strates. Additional advantages include the simplicity and gentleness of immobilization, allowing simultaneous immobilization of many enzymes acting in sequence. Among the disadvantages are possible enzyme leakage and no stabilization of enzyme activity is achieved by the immobilization.

4. Crosslinking

This method involves insolubilization of the enzyme by intermolecular crosslinking with a bi- or multifunctional reagent (e.g. glutaraldehyde) (1-3). Large, gelatinous aggregates are formed and considerable loss of enzyme activity is usually observed. Because of this and the difficulties in controlling intra- versus intermolecular crosslinking, this direct aggregation approach has found rather limited application. A second inert protein has been added to favor intermolecular crosslinking. Better activity yields can thus be obtained (47). By crosslinking of the enzyme + inert protein solution in the frozen state, porous, proteic copolymer particles have been obtained that allow continuous column operation (47). Hybrid methods involving crosslinking (preferably with glutaraldehyde) after adsorption to a solid support or after entrapment are however more frequent.

Intramolecular crosslinking has been reported to stabilize the conformation of many enzymes (a review can be found in ref. 48). This may result in increased enzyme stability after immobilization by covalent crosslinking. Derivatization of preformed solid supports with bi- or multifunctional reagents, where ideally one of the functional groups reacts with the support and the remaining groups with the enzyme is a common procedure for covalent attachment of enzymes (see next section).

5. Covalent attachment

This approach is the most thoroughly investigated and most frequently used method for immobilizing enzymes and affinity ligands. It involves the reaction between an aqueous biomolecule solution and a water-insoluble, functionalized (or "activated") support. For preparation of adsorbents for affinity chromatography covalent attachment is used almost exclusively. Two main factors are usually considered when choosing the method for covalent attachment: (1) the type of functional group on the enzyme or affinity ligand that should be used to form the covalent bond with the support and (2) the type of support desired. Different support

materials for covalent immobilization are discussed in the next section. The functional groups on proteins that have been most frequently utilized for covalent binding include α - and ϵ - amino groups, α -, β - and γ -carboxyl groups, phenol rings, sulfhydryl groups and imidazole groups. It is important that the functional group(s) on the enzyme essential for activity, for binding of the substrate, for allosteric control or for subunit interactions not be involved in the covalent attachment to the support. To minimize this risk, enzymes are often immobilized in the presence of substrate or competitive inhibitor. Similarly, immobilization of low molecular weight affinity ligands should not involve groups essential for binding to the enzyme or protein to be purified. Another important aspect is that the binding reaction can be carried out under conditions that are optimal for preservation of the biomolecule's structural integrity and activity, i.e. in the case of enzymes usually at near neutral pH, low temperature and in aqueous media.

To minimize the cost of immobilization, especially with expensive enzymes or affinity ligands, the method should also be efficient, i.e. most of the material should be immobilized. In the case of enzymes, it is preferable if the immobilization results in preparations that are generally more stable than their soluble counterparts. A number of cases where covalently immobilized enzymes have been shown to be more stable for storage (49), and to denaturation by heat (50-52), or denaturing agents (53) than the corresponding soluble enzymes have been reported. Intermolecular reactions of enzymes, such as autolysis of proteases or precipitation of enzymes, because of aggregation in high concentration of organic solvent (12) or at elevated temperatures (54), are obviously prevented by their covalent immobilization. Furthermore, dissociation of oligomeric enzymes can be avoided by covalent attachment via all subunits (55).

Noncovalent interactions between the enzyme and the support as well as a more favorable microenvironment compared with the soluble enzyme may exert a stabilizing effect on the structure of the immobilized enzyme. Furthermore, the chemical modification of the enzyme's primary structure, that occurs during covalent immobilization, may result in increased stability (48).

Upon immobilization of enzymes, that usually contain many reactive side chains per molecule, several covalent bonds to the support may be established per molecule depending on the degree of activation. This multipoint of attachment has been advocated to stabilize the conformation of some enzymes against denaturation by heat or urea (50-53), and to facilitate "freezing" of enzymes in a certain conformation (14). On the other hand, as with adsorption to hydrophobic supports, too many reactive groups on the support may lead to lowered activity of the enzyme, because of the increased risk of active site modification or tertiary structure distortion. Furthermore, loss of enzymatic activity can be due to other factors than structural, such as the loss of active site metal ions, and then immobilization is not expected to increase stability.

Sometimes, a very low level of reactive groups on the support is desired and the method used should allow reproduction of activation in such cases. Thus, in studies of the aggregation behavior of multimeric enzymes, covalent binding via one subunit may be desired, requiring a support with very few reactive groups (56).

The numerous reactions that have been applied for covalent attachment of biomolecules can be grouped as follows:

1. Amide bond formation or acylation reactions
2. Arylation
3. Alkylation
4. Diazotization
5. Isourea or carbamate formation
6. Miscellaneous

Immobilization via covalent attachment usually involves two steps: (1) introduction of reactive groups on the support (activation); (2) reaction of the biomolecule with the activated support (coupling). Various activated carriers are commercially available, which makes immobilization by covalent attachment facile. Some of the more common techniques for covalent attachment are described below. For detailed reviews see refs. 1-3, 15-18.

5.1. Amide bond formation

Examples of reactions in this category are the carbodiimide mediated condensation of an amino and a carboxyl group, the reaction of acylazides with amines and the reaction of acid chlorides with amines.

In addition to these reactions many others have been employed for amide bond formation (1-3,16). There is one report on the use of proteolytic enzymes for attachment of N-protected amino acids to silica-bound leucinamide (57).

The carbodiimide reaction is convenient for coupling of biomolecules via either amino or carboxyl groups to carboxyl or amino group containing supports, respectively. Several carbodiimide derivatives are available, both water soluble and water insoluble. For coupling of proteins an aqueous solution of EDC (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide) is added to enzyme and support. The reaction is rapid and efficient coupling, even with high pK_a aliphatic amines, usually takes place at pH 5-8 in some hours and at 4°C. A colorimetric method for quantitation of unreacted carbodiimide during coupling have recently been published (58).

Rearrangement of the initially formed O-acylisourea derivative is a common side reaction in carbodiimide mediated condensations, leaving hydrophobic acylureas on the carboxyl containing supports. To avoid this, the carrier is reacted in an organic solvent with DCC (N,N'-dicyclohexyl carbodiimide) and N-hydroxysuccinimide ester resulting in formation of a N-hydroxysuccinimide derivatized carrier (59). This

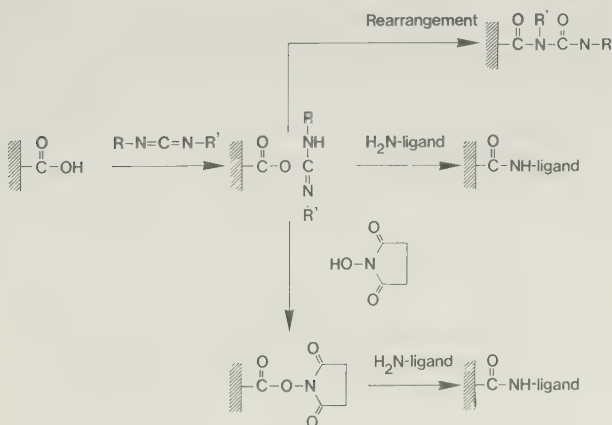


Fig. 1. Coupling of biomolecules via carbodiimide activation of carboxylic supports.

type of activation results in rapid amide bond formation with aliphatic or aromatic amino groups at pH 5-8.5 with almost no side reactions.

Another condensating agent, EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline), has been advocated to be favorable over the carbodiimides, being less toxic and resulting in formation of less by-products during the coupling (60).

Upon coupling of proteins with the peptide bond formation methods, sulfhydryl groups and aromatic hydroxyl groups can react in addition to amino groups (3,59). This results in formation of a number of labile bonds in addition to the stable amide bonds. Besides possible side-reactions, charged carboxyl or amino groups often remain on the support after amide formation, which may lead to nonspecific effects in affinity chromatography and changed pH-optima for immobilized enzymes.

5.2. Arylation

Synthetic polymers substituted with 3-fluoro-4,6-dinitrophenyl groups react with nucleophilic groups on proteins resulting in nucleophilic substitution of fluorine in the aromatic ring (61). This reaction is slower than the acylation reactions described above and a high pH is preferably used, i.e. pH 8-10.

Hydroxyl group containing polymers, mainly polysaccharides, have been activated with 2,4,6-trichloro-s-triazine (cyanuric chloride) or 2,4-dichloro-triazine derivatives at pH 9-11 and 20°C resulting in triazine activated supports (62). The activation step has also been carried out in organic solvents with an organic base to minimize hydrolysis of triazine reagents.

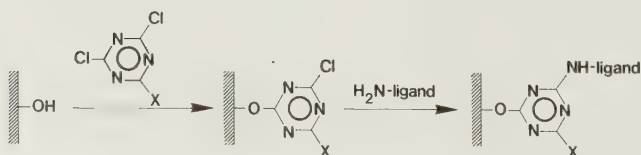


Fig. 2. Coupling of biomolecules to hydroxyl supports activated with 2,4,6-trichloro-s-triazine or 2,4-dichloro-s-triazine derivatives.

Coupling of proteins is carried out at pH 7-8 with cyanuric chloride activated supports and at pH 8-10 with dichloro-triazine activated carriers.

Polysaccharide supports have also been activated with p-benzoquinone (63). The advantage with this method is that efficient coupling of enzymes with good retention of activity takes place over a broad pH-range (3-10). Thus, the choice of pH for coupling depends on the stability and kind of biomolecule to be immobilized. However, undesirable side reactions are obtained, indicated by the color of the products.

The nucleophilic groups involved in coupling of proteins by arylation is thought to be mainly sulfhydryl, amino and phenolic hydroxyl functions (3). The aromatic groups used in these methods might result in nonspecific adsorption effects (64).

5.3. Alkylation

Examples of reagents mediating alkylation of functional groups on biomolecules are bisoxiranes, organic sulfonyl chlorides and sodium periodate combined with a reducing agent (NaBH_4 or NaBH_3CN). Organic sulfonyl chlorides have been used for immobilization in paper I-V in this thesis and are described in more detail in sections III and IV.

Bisoxiranes such as 1,4-butanediol diglycidyl ether has preferentially been used for activation of polysaccharides (especially agarose) (65, 66). The activation is performed at extremely alkaline pH (0.3 - 1.0 M NaOH) which results in the reaction of ideally one of the electrophilic oxirane (epoxide) groups with a deprotonated hydroxyl group on the support. With agarose extensive crosslinking takes place during the activation. Oxirane groups can be introduced on glass/silica beads by silanization with oxirane containing trialkoxysilanes (67). The amount of

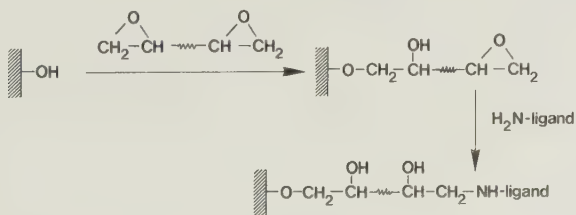


Fig. 3. Coupling of biomolecules to hydroxyl supports activated with bisepoxirane.

epoxide groups introduced is determined by reaction with thiosulfate ($S_2O_3^{2-}$) and the liberated hydroxyl ions are titrated with HCl (68).

For aliphatic amines the efficiency of the coupling reaction is very low at neutral pH but usually increases continuously up to pH 10-12. The method is therefore best suited for pH-insensitive biomolecules. Aromatic amines react however quite efficiently even at neutral pH (69). Thiols show a higher reactivity than amines. Aromatic hydroxyl, guanidino and imidazole groups in proteins may also react. Remaining epoxide groups can be removed after coupling with high concentrations of mercaptoethanol (18,70).

The advantages with the bisoxirane method are that stable S-C, N-C or O-C bonds are formed, an uncharged spacer can be introduced and the mechanical and chemical stability of the support increases because of the crosslinking obtained during activation. High concentration of bisoxirane may, however, lead to decreased porosity of the support due to extensive crosslinking. Furthermore, it is difficult to introduce more than 0.8 mmol oxirane groups/g dry agarose due to the crosslinking reaction (66), which, however, is enough for most applications.

The activation of supports containing vicinal diol groups, i.e. polysaccharides, glycerylpropyl-silica etc, by oxidation with sodium metaperiodate ($NaIO_4$) to aldehyde groups is a facile and rapid procedure (71,72). Coupling of amino groups at pH 5-7 results in rapid formation of Schiff's bases. These have to be reduced to yield stable N-C linkages. Reduction with $NaBH_3CN$ is often preferred over $NaBH_4$, because the former reagent is selective, reducing Schiff's bases much faster than aldehyde groups and is also much less prone to acid catalyzed hydrolysis than $NaBH_4$ (73,74). Thus, $NaBH_3CN$ can be present throughout the coupling reaction at pH 5-7, which has a favorable effect on the yield of coupled biomolecule. Reduction with $NaBH_4$ requires an alkaline pH (9-10) and is not suitable for ligands or enzymes

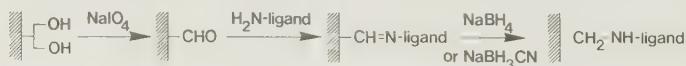


Fig. 4. Coupling of biomolecules to supports containing vicinal hydroxyl groups by periodate oxidation.

sensitive to alkaline conditions. An advantage compared with NaBH_3CN is that remaining aldehyde groups after coupling are effectively reduced with NaBH_4 .

A drawback with the periodate method is that some polysaccharide supports, such as highly porous crosslinked dextrans, tend to shatter after periodate oxidation. The degree of substitution obtained with agarose is generally low (59). Furthermore, the risk for reduction of sensitive biomolecules and of S-S bridges in proteins should not be overlooked in connection with the borohydride treatment (75,76). In a recent report, diol groups introduced as a side reaction during cross-linking of agarose were utilized for coupling of antibodies via periodate oxidation (77). With this method a satisfactory amount of bound IgG could be obtained.

In addition to oxidation by inorganic compounds, specific oxidation by an enzyme, D-galactose oxidase, of a polysaccharide (guaran) at position C-5 of the D-galactose residues, has been utilized for the introduction of aldehyde groups for subsequent reaction with amino group containing biomolecules (78).

5.4. Diazotization

Polymers containing aryldiazonium salt can easily be prepared by reacting covalently bound arylamines with nitrous acid (1-3, 16-18, 26, 27). The aryldiazonium group reacts rapidly at pH 8-9 with a wide variety of groups, such as the aromatic rings in tyrosine and histidine, with α - and ϵ -amino groups and at a slower rate with guanidino and indole groups. With the aromatic rings monoazo compounds are formed, while with the amino groups bisazo compounds, known as triazenes, are formed. Proteins are bound mainly via their tyrosine and lysine to diazotized supports. On reaction with dithionite, amine derivatives of the polymer and the biomolecule are formed (e.g. 3-aminotyrosine (79) and 6-aminopyridoxine 5'-phosphate' (80) can be prepared in this way).

Coupling of enzymes to diazotized electroneutral hydrophilic resins, (e.g. cellulose and starch) often results in low activity of the bound enzyme probably because of diazotization of essential hydrophobic regions in the interior of the protein (81). Diazotized hydrophobic resins are by the same rationale even more detrimental to most enzymes.

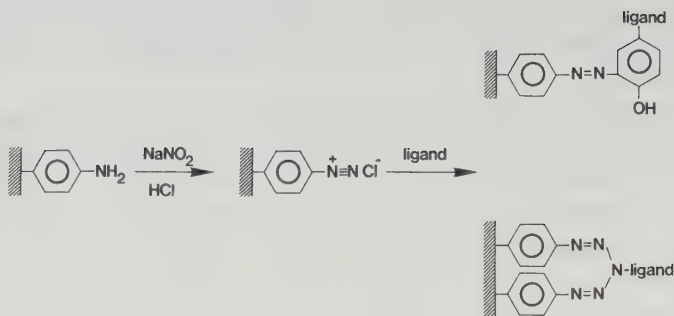


Fig. 5. Coupling of biomolecules to supports containing diazonium salts.

Higher activity is however often observed after coupling to diazotized polyelectrolyte supports (e.g. arylamine glass) (82). The method seems best suited for coupling of proteins with a high tyrosine content and of a hydrophobic character, such as alkaline phosphatase, which has shown to be coupled to CNBr -activated agarose with low retention of activity (3).

The method is also frequently used for the preparation of affinity adsorbents (16-18). For example, adenine nucleotides and pyridoxal 5'-phosphate derivatives have been rapidly immobilized via the C8 position in the adenine ring (82) and via the C4 position in the pyridoxal ring, respectively (80). This activation method introduces aromatic rings which can result in nonspecific adsorption effects (64).

5.5. Isourea and carbamate bond formation

The CNBr method is one of the most commonly employed methods for preparing immobilized enzymes and affinity ligands and is by far the most frequently used method for activating polysaccharides (29). The success of the method relies on the ease of activating the supports and the efficiency exerted in the coupling step. Proteins are optimally bound between pH 8 and 10 and aliphatic amines between pH 9 and 10 (84,85). At room temperature coupling usually is complete within 2 hours. Proteins are mainly bound through their amino groups, since the linkages obtained with sulphhydryl or tyrosinyl groups are readily reversible (86).

The mechanism of the CNBr activation of polysaccharides is thought to be very complex and is not exactly known (85). The reaction with

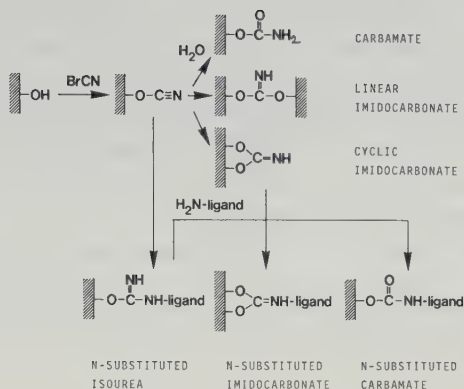


Fig. 6. A probable reaction scheme for $CNBr$ activation of supports containing hydroxyl groups and subsequent coupling of biomolecules.

$CNBr$ in 1 M K_2CO_3 is thought to introduce a relatively small percentage of highly reactive cyanate esters and less reactive cyclic imidocarbonates compared with a high percentage of almost inert carbamates and linear imidocarbonates (29, 85-90).

Upon reaction with amines the cyanate esters form N-substituted isoureas and the cyclic imidocarbonates form besides the isourea derivative, carbamate and imidocarbonate type linkages (85,89). Agarose only allows the formation of 6-membered cyclic imidocarbonates and is thought to form these rings less readily than dextran or cellulose, which allow formation of more stable 5-membered rings (85, 88). The extremely rapid hydrolysis of cyanate esters to carbamate and linear imidocarbonate at alkaline conditions makes the activation with $CNBr$ difficult to reproduce and results in low overall reaction yields as calculated on the amount of $CNBr$ used (usually less than 5%) (88, 90). It has recently been reported that the overall reaction yield and the percentage of cyanate esters formed can be substantially increased by making the activation at low temperature in a high percentage of organic solvent using an organic base as a catalyst (90).

The N-substituted isourea bond between the ligand and the support formed during coupling is slowly hydrolyzed in aqueous media, particularly at alkaline pH or in the presence of nucleophiles (e.g. amines or proteins) (87,91). In addition, the isoureas (pK_a about 10) are charged in the pH range usually used in affinity chromatography. This has been shown to lead to nonspecific effects due to ionic interactions

(17,18,92). It has been reported that the isourea bond causes more nonspecific binding than does the N-C bond formed by the oxirane method (93). Affinity ligands substituted with hydrazine groups have been synthesized to obtain uncharged linkages at neutral pH with the CNBr method (94). Stable linkages, probably of the guanidino type (support-NH-C(=NH)-NH-ligand) can be obtained on activation of supports containing amino groups with CNBr (29,95).

The carbamates, formed as a side reaction during activation and coupling, slowly release cyanate ions, which may cause inhibition of some enzymes (96). Another disadvantage is the high toxicity of the volatile CNBr reagent. To this end, the less volatile p-nitrophenylcyanate has recently been advocated to be preferable over CNBr (97). In this compound the p-nitrophenyl group acts as a leaving group in analogy with the bromine in CNBr.

Carbonyldiimidazole (98) and p-nitrophenylchloroformate (99) have recently been used for activation of hydroxyl group containing supports. Activation with these compounds is performed in an organic solvent yielding imidazole or p-nitrophenyl substituted carbamates. The reactivity of these carbamates is comparable with or slightly higher than that of epoxide groups. On coupling of amino ligands, the imidazole or p-nitrophenyl groups act as leaving groups and a noncharged N-substituted carbamate derivative is formed.

The advantage with these methods compared with the CNBr or p-nitrophenylcyanate methods is that a noncharged linkage is established with the support that is more stable than the isourea linkages formed by the cyanate reagents.

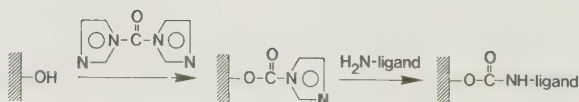


Fig. 7. Coupling of biomolecules to supports activated with carbonyldiimidazole.

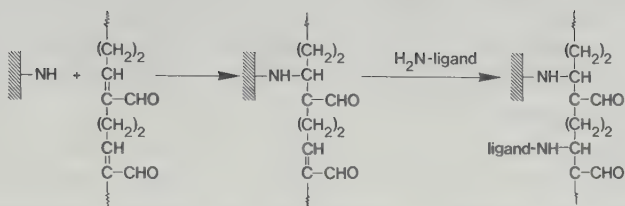


Fig. 8. A probable reaction scheme for coupling of biomolecules to supports activated with glutaraldehyde.

5.6. Miscellaneous

a) Bi- or multifunctional reagents

The most popular compound among these for covalent attachment is glutaraldehyde, which has been used for coupling of proteins to supports containing amino or amido groups (1-3). Immobilization with this reagent is simple and rapid. Activation is performed at pH 7 for about 1 h (a longer time is required for amide containing supports) at room temperature. The enzyme can be coupled in the pH range 5-9, the yield increasing with pH in this range.

The "glutaraldehyde" reagent largely consists of α , β -unsaturated oligomers of glutaraldehyde formed by aldol condensation (100). The mechanism of the activation and coupling is thus complex and probably involves both Schiff's base formation as well as a Michael type condensation reaction, in which an amine reacts by addition to an ethylenic double bond of the α , β -unsaturated glutaraldehyde reagent to give a stable N-C bond (100,101).

b) Four component condensation reactions (Ugi-reaction)

In this type of condensation one carboxyl, one amino, one aldehyde and one isocyanide group react to form an amide bond between the carboxyl and amino component (102). The aldehyde and isocyanide components appear as a side chain on the amide nitrogen. The reaction can be carried out at neutral pH and allows versatility in types of supports used

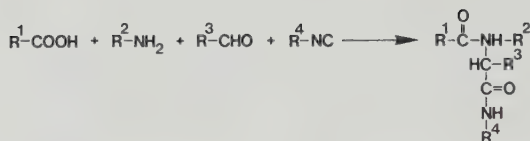


Fig. 9. Uqi-reaction

and groups in the enzyme coupled (103, 104). Thus, a protein can be bound via either carboxyl or amino groups to supports containing any of the four groups. Soluble amine (e.g. Tris) or carboxyl (e.g. acetate) compounds are added to steer the coupling reaction to the carboxyl or amino functions, respectively, on the enzyme when immobilizing to isocyanide or aldehyde containing supports.

c) Chelation

This method utilizes transition metal salts for linking an enzyme to a carrier (polysaccharides, nylon, glass beads, controlled pore titania, magnetic ironoxide, etc) (105, 106). The salts employed include TiCl_4 , TiCl_3 , SnCl_2 and ZrCl_4 . A number of procedures for immobilization by chelation have been developed, but principally the carrier is first treated with an aqueous metal salt after which it is washed and the enzyme added. The bonds formed are reversible and the enzyme will continuously leak off the support. The half lives of the bound enzyme depends on the system used, but is typically 10-30 days (107).

It is believed that activation of hydroxyl groups on the carrier and coupling of nucleophilic groups on the enzyme both involve replacement of water molecules from the transition metal complex (106). Covalently immobilized organic chelating agents have also been used (108). The desorption that occurs during operation or storage shows that weak interactions between the enzyme and the activated support are an important feature of the technique. Because of this, some authors have referred to it as an adsorption method rather than as partially covalent or chelation (106).

d) Coupling through carbohydrates on glycoproteins

Some glycoproteins are often immobilized with very low yields on coupling to various activated supports, probably because the amino acid side chains are shielded by carbohydrate residues. Such proteins can be effectively immobilized to supports containing amino groups after peri-

odate oxidation of the carbohydrate moiety (109, 110). Recovery of activity is often high, provided that the oxidized carbohydrate groups do not interact with active site region. Thus, a low recovery of peroxidase activity was reported after periodate oxidation of this enzyme. As described above (see section 5.3) the Schiff's base formed must be reduced with either NaBH_4 or NaBH_3CN to form a stable linkage. In a slightly modified scheme, periodate oxidized glycoproteins are reacted with low molecular weight diamines and then reacted with activated supports (111).

e) Specific covalent immobilization

Covalent attachment most probably leads to heterogeneously immobilized enzymes and, in addition, it is difficult to exactly assess which of the possible amino acid residues are modified by immobilization. Methods which specifically modify the enzyme would facilitate a better characterization of the immobilized preparation and would minimize the risk of changed intrinsic properties because of the sterical shielding by the support or modification of essential functional groups.

The immobilization of tryptophanase to agarose bound pyridoxal 5'-phosphate might serve as an example for such specific binding (80). The active site of one of the subunits in the tetrameric enzyme bound the coenzyme upon formation of a Schiff's base which was subsequently reduced by NaBH_4 . The other three subunits seemed to retain their activity. This method compared favorable with other covalent attachment methods. More than one of the subunits may however be affected.

In another similar example not using covalent immobilization, horse liver alcohol dehydrogenase was strongly biospecifically bound via one of its subunits, in the presence of isobutyramide, to a NADH analogue immobilized at a very low concentration on agarose (7). This allowed specific active site modification of the other subunit. Immobilized monoclonal antibodies directed against nonessential regions of enzymes might well be employed in the development of specific immobilization methods in the future.

f) Reversible covalent attachment

By using this approach it is possible to detach denatured enzyme by a specific reagent, regenerate the support and recharge with active enzyme. In one such method that utilizes thiol-disulphide interchange

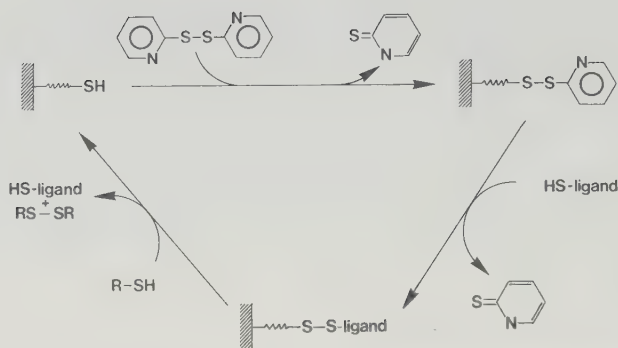


Fig. 10. Reversible immobilization of biomolecules by thiol-disulfide interchange reactions.

(Fig. 10) mercaptohydroxypropyl- ether-agarose is activated with 2,2'-dipyridyl disulfide, resulting in formation of 2-pyridyl disulphide hydroxypropyl-ether-agarose with concomitant release of 2-thiopyridone (112). The enzyme can then be efficiently coupled via their thiol groups at pH 7-8 and 4°C within 30-60 minutes. The 2-thiopyridone released during coupling can be used to monitor the reaction by measuring the absorbance at 343 nm. Release of the enzyme from the support is effected by reaction with a low molecular weight sulfhydryl reagent, such as mercaptoethanol or dithiothreitol. In addition to immobilization of enzymes, the thiol-disulphide exchange reaction has been utilized for covalent chromatography of thiol containing proteins and peptides (113, 114, for reviews see refs. 17, 18).

Enzymes not containing free thiol groups can be thiolated by reacting them with imidoesters containing thiol groups such as methyl-3-mercaptopropionimidate. By using the heterobifunctional reagent SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate), enzymes that do not contain thiol groups can be immobilized directly to supports containing thiol groups without prior modification of the support (115).

Some types of cleavable bonds other than disulphides have been utilized for reversible covalent immobilization. Among these are cleavage of azobonds by dithionite, which regenerate the arylamine groups on the support (79, 80, 84, 116), and cleavage of thioester bonds by reaction with strong nucleophiles, such as hydroxylamine (84, 117).

6. Supports for covalent attachment

6.1. General properties

Supports currently employed for immobilization are synthetic or natural in origin. They are based on organic or inorganic material, are chemically homogeneous or composites and/or are chemically modified. Examples of supports of single composition are glass, agarose and cellulose. Among the dual composition carriers are agarose-coated polyacrylamide, glycerylpropyl-coated silica and polyacrylic-coated iron particles. Crosslinked agarose and dextran represent chemically modified supports. In addition, distinctions are made between porous and compact ("pellicular") carriers, between xerogels (i.e. gels shrinking on drying, high pressures, changed polarity of solvent, etc) and aerogels (e.g. porous, inorganic supports) (1-3, 16-18). Macroreticular gels have a heterogeneous microstructure consisting of regions with highly aggregated polymer chains and regions with relatively large open spaces (e.g. agarose). Microreticular gels, on the other hand, have a relatively homogeneous distribution of polymer chains throughout the gel (e.g. crosslinked dextran) (16-18).

No generally optimal support exists for immobilization of enzymes or affinity ligands and the choice of support will depend on the application. Some factors that have to be considered are the physical form (granules, sheets, etc), the surface charge and hydrophilicity, the mechanical stability, the chemical and biological stability, the binding capacity, the pore size, the ease of derivatization and the cost and availability. In clinical applications where the support is in contact with blood or tissue, the support material should not cause any immunological response (wool support) or induce blood coagulation (glass support).

In some industrial applications of immobilized enzymes elevated temperatures are employed, requiring that a temperature stable support be used. Concerning enzyme stabilization it has been mentioned above (p.10), that multiple points of enzyme attachment may have a favorable effect on its conformational stability. To facilitate this, the support should allow a dense substitution with reactive groups. Supports with surfaces complementary to the enzymes tertiary structure would of course be ideal for obtaining maximum stabilization (39).

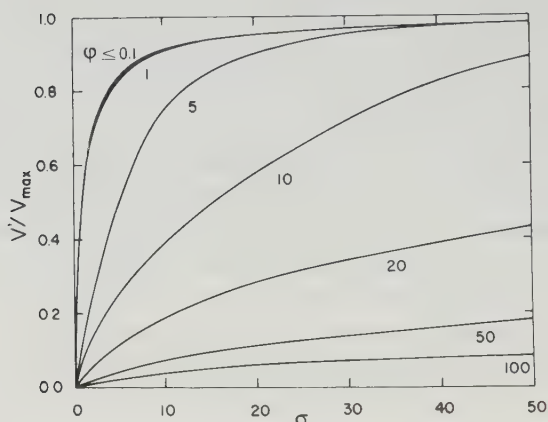


Fig. 11. Variation of the normalized overall rate with the dimensionless substrate concentration ($\sigma = S/K_m$) for different values of ϕ .

Another important criteria of the support is that it should allow rapid diffusion of the substrate to the immobilized enzyme. Similarly, limiting diffusion and nonuniform beads can be detrimental to the resolution in affinity chromatography increasing the peak widths of eluted substances (see section IV 3.3). In most cases immobilized enzyme kinetics are controlled by the diffusion of the substrate or product which may lead to erroneously high apparent K_m -values, more pronounced product inhibition and, if charged substrates or products are involved, changed apparent pH-optima compared with the soluble enzyme (1-3). Diffusional limitations are especially pronounced for large substrates and for supports of low pore size and for large particles. The loading of enzyme and its catalytic turnover rate as well as the stirring speed of enzyme gel suspension or the flow of substrate over a packed bed will naturally also influence the degree of diffusional limitations.

Generally, a reduction in the size of the support particle will lead to less internal diffusional resistances (i.e. diffusional limitations due to substrate transport in the carrier pores) (118,119). Thus, the substrate modulus for internal diffusion, known as the Thiele modulus, ϕ , is directly proportional to the particle radius, R (119):

$$\phi = R/3(V_{max}/K_mD)^{1/2}$$

The observed rate of V of an enzymatic reaction in a particle relative to the saturation rate, $V_{\max}$, at a given substrate concentration, S , is usually strongly dependent on ϕ (Figure 11). Another way to reduce diffusional limitations is to bind the enzyme to the surface of compact (nonporous) beads, but this leads to low loadings, unless very small particles ($< 1 \mu\text{m}$) are used.

Even in the absence of diffusional limitations a charged support, or introduction of charges during covalent attachment, may lead to apparent shifts in pH optima of the immobilized enzyme due to partitioning effects of charged solutes. Shifts by as much as two pH units have been observed (119). Partitioning effects due to hydrophobic or electrostatic interactions may cause shifts in Michaelis constants as well.

Nonspecific adsorption, caused by electrostatic and/or hydrophobic interactions, is detrimental to the performance of affinity columns (16-18). The use of supports that exhibit low nonspecific adsorption per se and which can be derivatized with retention of this property is therefore desired when designing affinity adsorbents.

From the first publications on covalent immobilization of enzymes, it was concluded that hydrophobic supports (e.g. polystyrene based) caused inactivation of enzymes upon attachment (120). Hydrophilic supports have since then been predominantly employed. Polyacrylamide and polysaccharide supports are hydrophilic and have a high binding capacity of protein because of their high surface area and pore size. They are easily derivatized with active groups for covalent attachment, show a low nonspecific adsorption and are available at a relatively low price. However, for continuous operation in enzyme reactors or affinity columns, the relatively low mechanical stability of these supports does not allow for high flow rates or pressure falls. Highly crosslinked agarose gels of high concentration (9-15%) have however been used at intermediate pressures (121). Another drawback of polysaccharides is the risk of microbial attack. In applications such as enzyme electrodes and membranes, the formability of organic polymers, such as polyacrylamide, is an advantage.

The rigidity of macroporous inorganic supports (e.g. glass, silica) makes them ideal for packed bed reactor applications and for high pressure affinity chromatography. The small, uniformly sized, silica particles that are commercially available give sharper peaks of eluted compounds than larger, less uniformly sized, particles (122). Consequently, a higher resolution and a more rapid separation is possible.

This has been utilized in high performance liquid affinity chromatography (HPLAC; refs 70, 123, paper V).

A drawback with these supports is the extensive nonspecific adsorption which occurs. However, this can be significantly reduced by coating the surface with a hydrophilic, electroneutral organic layer (18). As to the chemical stability, glass and silica based supports are slowly dissolved above neutral pH. These supports are more expensive than supports based on polysaccharides or polyacrylamide.

In this thesis, supports based on agarose and porous silica coated with glycerylpropyl groups have been predominantly used (paper I-V).

6.2. Agarose

Beaded agarose is the most frequently employed support material for affinity chromatography and is also among the most commonly used carriers for immobilization of enzymes. The chemical structure of agarose, a seaweed polysaccharide, consists of linear chains of alternating 3-linked β -D-galactopyranose and 4-linked 3,6-anhydro- α -L-galactose residues. Adjacent chains are arranged in rigid double helices that in turn are aggregated to regions of highly ordered structure (124). These regions contain 10 to 10^4 helices. Some of the polysaccharide chains effect crosslinking by participating in more than one of the ordered regions.

Three to four hydroxyl groups of the repeated disaccharide unit protrude outwards from the double helical structure and are presumably available for chemical substitution or hydrogen bonding with neighboring helices or the solvent. The core of the double helix is hydrophilic and thus can not act as a repository for hydrophobic substances, as is the case with the amylose helices in starch.

The popularity of agarose in affinity chromatography probably depends on the fact that it exhibits many of the properties of an ideal support: (1) It is easily derivatized by a number of methods for the covalent attachment of ligands, allowing a high density of substitution (more than 1 mmol per g dry weight); (2) it exhibits a very low nonspecific adsorption per se; (3) it forms a macroreticular network of high porosity, which allow easy accessibility to bound ligands throughout the beads, even for very large molecules (up to mol wt 20×10^6); (4) it is chemically and physically stable under conditions normally used in low pressure affinity chromatography; (5) the beads are spherical, which is beneficial for chromatographic performance.

The binding capacity for proteins is high. Thus, conjugates containing more than 300 mg of covalently bound low molecular weight protein (chymotrypsin, STI) per g of dry conjugate has been prepared (125, paper III). The binding capacity of agarose in the gel state is however considerably lower, since the water content of the commercially available beads usually is 20 - 30 times the dry weight.

Drawbacks with agarose are, as mentioned above, that the beads are compressed during high pressure, are vulnerable to microbial attack and are commercially available in a nonuniform and relatively large size (60-140 μm , (126)). Also, the arrangement of helices in bundles can result in that most of low molecular weight affinity ligands are immobilized in the interior of these bundles, thus being inaccessible for interaction with macromolecules (32).

6.3. Glycerylpropyl-silica

Porous silica is available in pore sizes ranging from 50-4000 Å and in particle sizes down to 2 μm . The silanol groups on the surface of porous silica are slightly deprotonated at neutral pH. This causes nonspecific adsorption of several proteins by charge-charge interactions (31). Hydrogen bonding may also contribute. Coating of the silica surface with glycerylpropyl groups has been shown to minimize such nonspecific adsorption (127). Glycerylpropyl-silica was initially utilized as a support for high pressure gel chromatography of proteins, but was soon adopted as a suitable carrier for HPLC (70, 123).

Glycerylpropyl-silica is prepared by silanization with γ -glycidyloxypropyltrimethoxysilane with subsequent hydrolysis of epoxide groups to diol groups at acidic pH (Fig. 12). Alternatively to hydrolysis, the epoxide groups can be utilized for direct coupling of biomolecules (see section IV 3.2).

The trimethoxysilane is very reactive towards water. To avoid attachment of the silane to the surface as a polymeric layer the reaction is therefore carried out under anhydrous conditions (128).

Modification with 2-2.5 μmol glycerylpropyl groups per square meter is characteristic of a monolayer (128). The surface area of porous silica decreases with increasing pore size from about 200 m^2 per g of dry 100 Å silica to about 20 m^2 per g of dry 1000 Å silica. Thus, about 400 and 50 μmol diol groups per g can be introduced on 100 and 1000 Å silica, respectively. Despite this, the size of proteins (mostly 30-100 Å

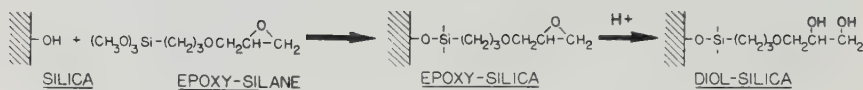


Fig. 12. Preparation of glycerylpropyl-silica.

in diameter) makes 100 Å silica (about 70 Å pores after the coating with glycerylpropyl groups) less suitable for immobilization of enzymes or for affinity chromatography. There is, however, a variation of the pore size around the mean value and this allows for coupling of higher amounts of large proteins to the small pore size silica than that expected if only the outer surface was available (70).

Silica with 300 Å pores has practically the same surface area as the 100 Å variant and should therefore be useful for applications where high capacity are desired. Thus in paper V, 280 mg soybean trypsin inhibitor was bound per g of dry silica, corresponding to about 90 mg protein per g of wet silica.

Large pore silica (300 - 1000 Å) is presently available in particle size down to 10 µm, which makes them suitable for HPLAC applications.

III IMMOBILIZATION USING ORGANIC SULFONYL CHLORIDES

1. General properties of sulfonate esters

Sulfonate esters, which have the general formula $R^1SO_2OR^2$, have been used in organic chemistry for over one hundred years (129). Their chief applications have been in substitution and elimination reactions where the sulfonate anion, $R^1SO_3^-$, functions as a leaving group. The reactivity is strongly influenced by the nature of both R^1 and R^2 . Thus, the relative solvolysis rates in aqueous ethanol for p-toluenesulfonate (tosylate), p-nitrobenzenesulfonate, 2,2,2-trifluoroethanesulfonate (tresylate; $CF_3CH_2SO_3R^2$) and trifluoromethanesulfonate (triflate; $CF_3SO_3R^2$) have been reported to be 1:20:100:4000 (130, 131)., When R^2 is alkyl they are easily hydrolyzed, but for R^2 = aryl, hydrolysis is much slower.

The preparation of sulfonate esters is simple and usually involves reaction in an anhydrous solvent of an alcohol with a sulfonyl chloride and a base (usually pyridine). Primary alcohols react much more rapidly than do secondary alcohols. Actually it is possible to prepare the primary tosylate exclusively from a compound containing both primary and secondary hydroxyls (129).

Alkyl sulfonate esters are used as powerful alkylating agents, alkylating nucleophiles of great diversity, such as halides, alkoxides, phenoxides, mercaptides, and amines. Even very poor nucleophiles, such as benzene, have been reported to be alkylated with the highly reactive trifluoromethanesulfonates (132). Most of the reactions between primary alkyl sulfonates and good nucleophiles can be understood according to the S_N2 mechanism paradigm, where the sulfonate anion acts as a leaving group (Fig. 13). Cleavage of S-C or S-O bonds are very uncommon. Thus, they resemble alkyl halides as alkylating agents. But, the reactivity spectrum of the various alkyl sulfonates is much broader and their preparation from alcohols is simpler. In fact, sulfonate esters of sugars may be used for the preparation of sugar halides (the halide ion acting as nucleophile) (133).

The reactivity of alkyl tosylates and alkyl bromides is roughly the same, but tosylates have been reported to give much more of desired substitution relative to elimination than the corresponding bromides (129).

This thesis demonstrates the utility of sulfonate esters for covalent immobilization of biomolecules to supports carrying hydroxyl groups. Among the potential advantages of using organic sulfonates for immobilization are: (1) the activation procedure is relatively simple; (2) the conversion of hydroxyl groups into good leaving groups allows a covalent bond to be formed between the support carbon carrying the hydroxyl group and the nucleophile on the biomolecule, without the introduction of additional groups; (3) the possibility of choosing from a wide variety of R groups gives the sulfonates a broad reactivity spectrum, allowing one to find the suitable derivative for the particular biomolecule to be immobilized (Fig. 13).

In paper I-V, *p*-toluenesulfonyl chloride (tosyl chloride) and 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) were predominantly used. Three other compounds have been studied in a more preliminary fashion, i.e. *p*-nitrobenzenesulfonyl chloride, *o*-nitrobenzenesulfonyl chloride and trifluoromethanesulfonic anhydride (unpublished). Recently, the use of colored sulfonyl chlorides, such as dansyl chloride, for activation of hydroxylic supports was reported (134). The advantage of these latter compounds is that activation and coupling can be followed visually.

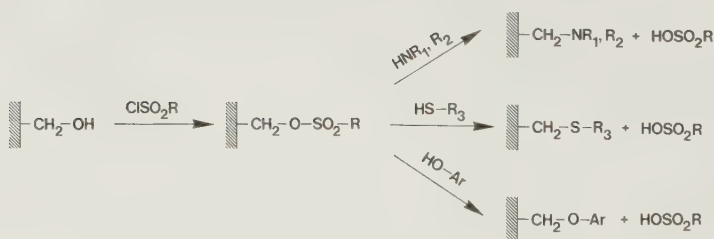


Fig. 13. Coupling of biomolecules to hydroxylic supports using organic sulfonyl chlorides.

2. Reaction of supports with organic sulfonyl chlorides (activation)

The activation is performed in a water-free solvent in order to avoid hydrolysis of the sulfonyl chloride. Pyridine is added to neutralize liberated HCl. Higher yields are obtained when using dried solvents rather than the commercially available analytical grade. Complete anhydrous conditions are, however, unnecessary and in this work molecular sieve 4 A (Merck) has been used as a drying agent (30 g per liter solvent).

In the first two papers in this thesis, dioxane was preferentially used as solvent. Later on, acetone was used in activation of solid supports as it is cheaper and less toxic. The reaction is performed in a well ventilated hood. In addition to the activation of agarose and glycerylpropyl-silica, that will be described here, cellulose, hydroxyethyl methacrylate and glycophas glass have also been activated with tresyl chloride (paper III). In addition, water soluble tresyl-PEG (poly(ethylene glycol), mol wt 4000 or 6000) has been prepared (135). This compound should be suitable for preparation of PEG-ligand derivatives used in membrane reactors (136), in affinity partitioning (20, 137) or in partition affinity ligand assay (138). PEG activated with cyanuric chloride (139) or carbonyldiimidazole (140), has been applied for covalent modification of several enzymes so as to render them nonimmunogenic. Tresyl-PEG is probably more reactive than the PEGs activated as above, thus allowing modification under milder conditions.

Recently, the sulfonate method has been used for immobilization of proteins (IgG, ConA, lactate dehydrogenase) to glycerylpropyl-coated silicon plates (141).

a) Activation of agarose with tresyl chloride

In a typical procedure, 5 g wet Sepharose 4B (Pharmacia, Sweden) is washed successively with 50 ml of each of the following: 30:70 and 70:30 of acetone:water (V/V), acetone p.a. (twice) and three times with dried acetone. The gel is then transferred to a dried beaker containing 1.5 ml of dry acetone and 75 μ l of dry pyridine (dried with a molecular sieve). During magnetic stirring, 50 μ l tresyl chloride is added dropwise. After 10 min at room temperature, the gel is washed twice with 50 ml of each of the following: acetone p.a., 30:70, 50:50 and

70:30 of 5 mM HCl:acetone p.a. (V/V) and finally with 5 mM HCl and stored at 4°C until used. The amount of introduced tresyl groups is determined on the freeze-dried gel by elemental analysis for sulfur. This activation procedure will introduce about 450 μmol tresyl groups per g of dry gel (135).

b) Activation of glycerylpropyl-silica with tresyl chloride

In a typical activation, 4 g of the dry support is washed three times with 50 ml each of dried acetone. The moist gel (about 10 g) is added to a dry beaker containing 5 ml of dry acetone and 260 μl of dry pyridine with magnetic stirring. Tresyl chloride (180 μl) is added to the suspension. After 15 min at 0°C the gel is washed as described for agarose. This procedure will introduce about 130 μmol tresyl groups per g of dry silica (300 Å pores) (paper V). If not used directly for coupling, the gels are preferably washed with water, 50:50 water-acetone and acetone, dried and stored in desiccator until required.

c) Activation of agarose with tosyl chloride

The procedure for activation with tosyl chloride is similar to those described above. Agarose is transferred to dry acetone as above (see a). Tosyl chloride (0.6 g) is dissolved in 3 ml of dry acetone, and 8 g of moist Sepharose CL 6B is added. After addition of 1 ml of dry pyridine, the reaction is continued for one hour at room temperature. After transfer to 5 mM HCl the gel is ready for use. This procedure will introduce about 0.7 mmol tosyl groups per g of dry agarose. The degree of substitution with tosyl groups can be determined by elemental analysis of sulfur or by measuring the difference in absorbance between tosylated agarose and untreated agarose at 261 nm ($\epsilon = 480 \text{ M}^{-1}$, paper I and II).

Table 1 and 2 shows the amount of tresyl and tosyl groups, respectively, introduced when employing varying amounts of the respective sulfonyl chloride. The data are extracted from paper I, III and IV. The amount of tosyl and tresyl groups introduced was, in most cases, directly proportional to the amount of sulfonyl chloride used.

Table 1. Activation of agarose and glycerylpropyl-silica with tresyl chloride.

Support	Amount of tresyl chloride used mmol /g dry support	Amount of tresyl groups introduced mmol /g dry support	Yield %
Sepharose 4B	0.70	0.11	16
Sepharose 4B	2.70	0.45	17
Sepharose 4B	4.50	0.80	18
Sepharose 4B	9.00	1.30	15
Sepharose CL-6B	2.00	0.45	23
Sepharose CL-6B	4.00	0.98	25
Sepharose CL-6B	6.00	1.10	18
Diol-silica 300	0.16	0.05	31
Diol-silica 300	0.41	0.13	31
Diol-silica 300	2.45	0.40	16

Table 2. Activation of agarose (Sepharose CL 6B, Pharmacia) with tosyl chloride.

Tosylation time min	Amount of tosyl chloride used mmol/g dry agarose	Amount of tosyl groups introduced mmol/g dry agarose
10	13	0.70
30	13	1.10
60	13	1.40
60	26	1.97
60	6.5	0.70
60	2.6	0.20

As expected, tresyl chloride forms sulfonates much more readily than tosyl chloride does. Thus, 4 mmol tresyl chloride forms 0.98 mmol tresylates per g of dry Sepharose CL 6B in 10 minutes, whereas 13 mmol tosyl chloride and 30 minutes reaction is needed for a similar degree of substitution. Still the tosyl chloride activation is conveniently rapid for the introduction of an appreciable amount of tosyl groups. Thus, with 13 mmol tosyl chloride, 1.4 mmol tosyl groups per g of dry agarose (i.e. about 70 μmol per ml of settled Sepharose CL 6B) is introduced after 60 min reaction. Such a high degree of substitution is more than adequate in most applications. Furthermore, both the introduction of tosyl groups during the activation as well as the release of toluenesulfonic acid during the coupling can be followed by measuring the UV-absorbance.

The 300 Å pore glycerylpropyl-silica used in Table 1 contained about 400 μmol diol groups per g of dry silica. On reaction with tresyl chloride, 400 μmol tresyl groups per g of dry silica could be introduced. The same observation was made with 100 Å and 1000 Å pore silica. It can however not be definitely concluded that all diol groups contained one tresyl group, as secondary hydroxyls may also react.

3. Coupling of affinity ligands and enzymes

The procedures for coupling biomolecules to tresylated or tosylated supports are very similar to those for coupling to supports activated with CNBr or bisoxiranes, respectively. A number of examples are given in papers I-V. Generally, the buffer used for coupling should not contain strong nucleophiles that might react with the activated support and thus compete with the biomolecule during coupling. Phosphate, carbonate and hepes buffers were used in this work. Since sulfonic acid is liberated during coupling the pH will decrease if the buffer concentration is too low. Usually, 0.1 - 0.2 M buffer is sufficient to minimize the change in pH.

An advantage in the coupling step over CNBr-activated supports is that tosylated or tresylated supports can be stored for several weeks in 1 mM HCl at 4°C without losing more than a few per cent coupling capacity (as tested by low molecular weight compounds e.g. γ -aminobutyric acid). Even at neutral pH the stability against hydrolysis is quite high. Thus, after storage at 4°C and pH 7.5 overnight, the decrease in tresyl groups from agarose was only about 5%. Freeze-dried tresyl-silica, have been stored for one year in a desiccator without losing coupling capacity.

Coupling to tresylated supports is efficient at near neutral pH and at 4°C (Table 3). Because of the high reactivity of tresylated supports, a low activation level (i.e. 150 - 300 μ mol tresyl groups per g of dry agarose or 50 -100 μ mol per g of dry silica (100 - 300 Å pores)) still allows efficient immobilization. Thus, 90% of added protein A (5 mg/g) was bound to agarose activated with 150 μ mol tresyl groups per g of dry carrier (142). If, however, very high protein loading of the carrier is desired the activation level has to be increased. By doing this, virtually all of the available surface on glycerylpropyl-silica could be covered with protein as calculated from the dimensions of the protein (Table 3, see paper V).

The same protein, soy bean trypsin inhibitor (mol wt 21500), was bound in even higher amounts to tresyl-agarose (i.e. 366 mg/g dry carrier) at 4°C and pH 8.5 (paper III). Most likely, much higher amounts of high molecular weight proteins can be attached. As an indication of this, tetrameric concanavalin A (mol wt 110 000) was bound in almost 100% yield to tresyl-agarose at a similar loading (360 mg/g carrier).

The effect of pH and reaction time on coupling of STI at 4°C to tresyl-agarose was investigated in paper III. The optimal pH was around pH 9.5, but the yield decreased by only about 10 % when coupling was instead carried out at pH 7.5. At pH 8.5 the coupling was almost complete in 1.5 hour. The pH and temperature dependence as well as coupling capacity of tresyl-agarose for amino group containing ligands seems to be very similar to that of CNBr-activated agarose (unpublished, see also ref. 125).

As mentioned above (p. 29) alkyl sulfonates are used for alkylating a number of different nucleophilic groups, such as thiols, amines and phenolic hydroxyls. The difference in reactivity of various nucleophiles towards alkyl sulfonates can be qualitatively understood in terms of hard soft acid base principles (143, 144). In paper II it was shown that thiol groups (soft) reacts more readily than amino groups (hard) with tosyl-agarose (the electrophilic carbon in alkyl sulfonates is considered soft). This also holds for tresyl- agarose (unpublished observation). For both type of supports, the yield of bound ligand with thiol group containing low molecular weight compounds is about twice as high as with the corresponding amino group compounds. The slow hydrolysis of agarose sulfonates might be qualitatively understood, if one considers the "hardness" of the oxygen in water or in hydroxyl ions.

Table 3. Coupling of biomolecules to tressyl chloride activated supports

Support	Amount of tressyl groups on the activated support mmol/g dry support	Ligand	Time for coupling h	pH	Bound ligand mg/g dry support	Yield %
Sepharose 4B	0.15	Protein A	15	8.5	70	90
Sepharose 4B	1.30	Concanavalin A	15	7.5	360	97
Sepharose 4B	1.30	STI	15	7.5	195	68
Sepharose 4B	1.30	STI	15	8.5	211	74
Sepharose 4B	1.30	STI	1.5	8.5	200	70
Sepharose CL-6B	1.30	N ⁶ -(6-aminohexyl)- 5'-AMP	15	8.2	87	32
Sepharose CL-6B	1.10	Trypsin	15	8.2	124	70
Cellulose	0.50	Trypsin	15	8.2	61	87
Diol-silica 1000	0.06	STI	20	8.0	46	92
Diol-silica 300	0.13	STI	20	8.0	115	33
Diol-silica 300	0.40	STI	20	8.0	280	80
Diol-silica 300	0.40	STI	1	8.0	220	63
Diol-silica 100	0.45	N ⁶ -(6-aminohexyl)- 5'-AMP	20	8.0	13	80

a) All couplings were performed at 4°C, except for Protein A and for the AMP-analogue to diol-silica, which were made at room temperature.

b) Soybean trypsin inhibitor

c) Glycerylpropyl-silica (10 μm).

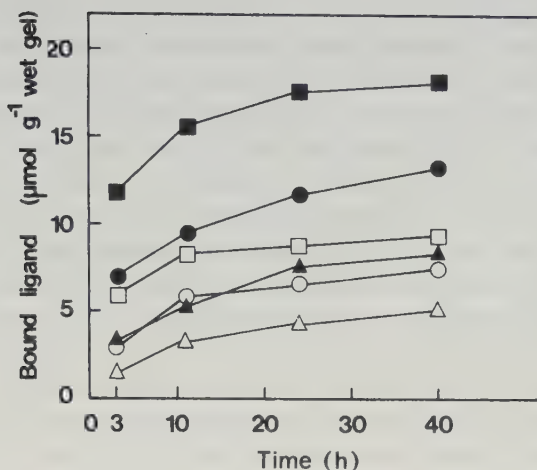


Fig. 14. Dependence of coupling time and temperature for the coupling of β -alanine at pH 10.6 (open symbols) and of β -mercapto propionic acid at pH 10 (closed symbols) to tosyl-Sepharose Cl 6B. (Δ) 4°C; ($\circ$) 22°C; ($\square$) 40°C. The ligand concentration was 0.4 M.

Although less reactive than its tresylated counterparts at neutral pH and 4°C, tosylated supports can immobilize satisfactory amounts of affinity ligands at alkaline pH and at higher temperature (paper I and II). For most applications in affinity chromatography, 1-10 μmol low molecular weight affinity ligand immobilized per g swollen agarose is considered optimal. As shown in paper II, 15 μmol mercapto propionic acid and 8 μmol β -alanine per g of settled agarose are easily introduced (Fig. 14). Very high amounts of ligand can be bound when coupling takes place in DMF. About 40 μmol hexylamine per g of settled agarose was thus introduced, when the ligand was coupled in this solvent. Such high amounts of immobilized ligand are beneficial in affinity chromatography when weak forces are utilized, e.g. hydrophobic interaction chromatography (17,145,146). The construction of uncharged hydrophobic affinity adsorbents from thiol compounds and tosyl-agarose, would probably be more facile than the oxirane chemistry that is commonly practiced today (17,18, 147).

Tosyl-agarose coupled enzymes in good yields at room temperature and at alkaline pH (paper I). HLADH was found to bind in high yields (60%) at pH 7.5 with the same retention of activity as when bound to CNBr-activated agarose. This enzyme couples much more efficiently at lower pH than for instance STI, which can be attributed to the many (28) free -SH groups on the surface of the HLADH molecule (148).

Summarizing, supports activated with tresyl chloride efficiently bind proteins and affinity ligands under mild conditions. Tresyl chloride is therefore the reagent of choice for immobilization of temperature and alkaline pH sensitive biomolecules. Tosylated supports are suitable for immobilizing cheaper, less sensitive, biomolecules. Tosyl chloride is inexpensive and the introduction/replacement of tosyl ester can be conveniently followed by UV-measurements. Compared with the bisoxirane method, no crosslinking or spacer is introduced which may or may not be advantageous. Tosylation is, however, faster and can easily be followed and a higher substitution with reactive groups can be obtained (cf. paper II).

In addition to thiol and amino groups, it has been shown that imidazole and phenol groups can bind to tosyl- and tresyl-agarose (paper II and unpublished). Amino acid analysis of bound horse liver alcohol dehydrogenase (HLADH) indicated that cysteine, lysine, arginine, tyrosine and histidine side chains had reacted with tresylated silica (unpublished).

This broad range of reactive nucleophiles can be a great advantage when a very "tight" binding between protein and support is desired, e.g. for stabilization of protein conformation (cf. section IV 1). Furthermore, one is not restricted to spacers carrying amino groups when constructing affinity ligands. By using thiol spacers, uncharged S-C bonds are obtained. With CNBr-activated supports on the other hand, one is restricted to ligands containing amino or hydrazine groups, as sulfhydryl, imidazole and aromatic hydroxyl groups form linkages which are easily hydrolyzed (86). Consequently, proteins rich in these latter residues are expected to bind more efficiently to tresyl-agarose.

4. Some properties of immobilized enzymes

A number of enzymes have been immobilized to tresylated supports (paper III-IV, 149, 150). In paper III trypsin (10 mg/g wet carrier) was bound to tresylated agarose, cellulose and glycerylpropyl-silica with 70, 87 and 80 % yield, respectively, at 4°C and pH 8.2 (Table 3). The retained apparent activity relative to soluble enzyme was highest for glycerylpropyl-silica (63%), probably because the small particle size of the carrier leads to less restricted diffusion of substrate. Hexokinase was bound with 53 % yield at pH 7 and 4°C. The retained apparent activity was in this case 25% relative to the free enzyme. These values for trypsin and hexokinase, immobilized to tresyl-agarose, are in agreement

with results obtained when the enzymes were bound to CNBr-activated agarose (unpublished). No competitive inhibitor was present during the coupling of these enzymes and therefore autolysis (trypsin) and active site modification might, in addition to diffusion limited kinetics, contribute to the decreased activity of these enzymes after immobilization.

T₄ DNA ligase (149), HLADH (150) and chymotrypsin (paper IV) have also been coupled in high yields to tresyl-agarose. The immobilized T₄ DNA ligase could be repeatedly used for joining DNA fragments and remained active for over 3 months (149). The retained activity of this sensitive enzyme after immobilization was strongly dependent on the degree of activation of the support. The reproducibility of the activation even with low amounts of tresyl chloride, was an important advantage in this application. Chymotrypsin immobilized to tresyl- Sepharose CL 4B has been used for repeated enzyme catalyzed peptide synthesis in aqueous-organic solvent mixtures (paper IV, see section IV 2).

A thorough characterization of immobilized HLADH coupled to tresyl chloride activated glycerylpropyl silica, was made in paper V. HLADH is one of the best characterized enzymes both kinetically and structurally (148, 151). It is a dimeric, two substrate enzyme with a molecular weight of 80 000. The enzyme has three isozymic forms, usually termed EE, ES and SS (148). Commercially available preparations contain the EE form and small amounts (2-5%) of the ES dimer (148). There exists strong experimental evidence that both subunits in the EE isozyme are kinetically equivalent (152). HLADH catalyzes oxidoreductions of the type $H^+ + NADH^+ + C=O \rightleftharpoons NAD^+ + C(H)OH$. The broad structural specificity towards different alcohols or aldehydes/ketones, the high enantiomeric specificity and prochiral stereospecificity makes the enzyme well suited for organic synthesis and ample examples of this can be found in the literature with the soluble enzyme (153, 154).

Removal of the catalytic zinc atom leads to inactivation and changes the coenzyme binding characteristics (155). Modification of lysine 228 ("Plapps lysine"), which is situated in the coenzyme binding domain, weakens the binding of the coenzyme (156). In paper V HLADH was immobilized in its ternary complex conformation with NADH and isobutyramide since this is known to protect the soluble enzyme against release of catalytic zinc (157) and against modification of lysine 228 (156).

As mentioned previously, a direct quantitation of the intrinsic activity remaining after immobilization to agarose is often difficult to obtain because of the diffusional hindrance caused by the relatively large agarose particles (60-140 μm). A better quantitation would be possible by immobilization to smaller particles (cf. Section II 6.2). Glycerylpropyl-silica with 1000 Å pores (allowing easy penetration of enzyme and substrates) is available down to 10 μm particle size. As shown in Table 4, virtually complete retention of the HLADH activity added was obtained after immobilization to such silica particles activated with tresyl chloride, despite quite a high loading of enzyme. Obviously, no diffusion limitations exist at the assay conditions employed, since the activity increased proportionally with loading of enzyme on the support. Coenzyme binding site determination with isobutyramide and radioactive NADH, indicated virtually complete retention of these sites after immobilization. A blank silica, tresyl-silica treated with Tris-HCl, did not bind any NADH. Since the rate limiting step for free HLADH catalysis is the dissociation of the coenzyme, the retained coenzyme binding sites and specific activity indicate that the dissociation rate for NADH from the immobilized enzyme is as high as for the soluble enzyme.

K_m for ethanol and acetaldehyde were very similar to that of the soluble enzyme (0.4 and 0.3 mM, respectively) at pH 7.5.

The dissociation constant of the immobilized enzyme-NADH complex was very similar to that reported for the free enzyme (0.6 μM , kinetic method at an ionic strength of 0.6 M and pH 7 (158); 1.2 μM , equilibrium measurements (155)). The dissociation constant was determined by plotting the amount of bound radioactive NADH to the immobilized enzyme according to Scatchard (Fig. 15). Furthermore, the linear Scatchard plots indicated that the vast majority of the coenzyme binding sites were homogeneous. A small population of weaker binding sites was, however, inferred from the independent coenzyme binding site determination with radioactive NADH + isobutyramide (K_d is about $10^{-8}\mu\text{M}$ for NADH and soluble enzyme in this ternary complex (159)). The result of this measurement is indicated by arrows in Figure 15.

Table 4. Coupling of horse liver alcohol dehydrogenase to tresyl chloride activated glycerylpropyl-silica.

Amount of tresyl chloride used in the activation ($\mu\text{mol/dry silica}$)	Amount of enzyme added (mg/g dry silica)	Amount of enzyme bound (mg/g dry silica)	Relative activity of bound enzyme compared with free enzyme (%)	Retention of coenzyme binding sites (%)	Dissociation constant NADH (μM)
100	21	12	100	100	0.87
300	21	21	95	97	0.80

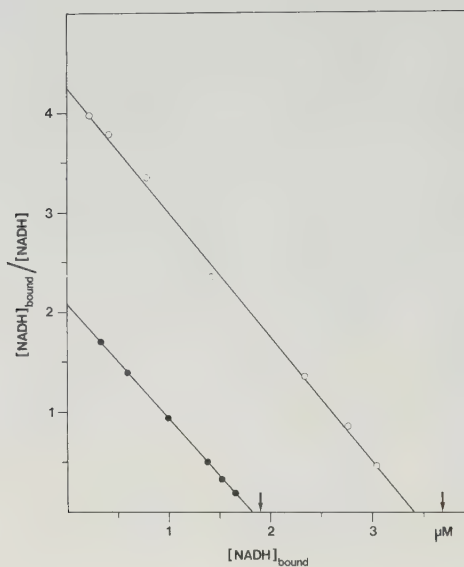


Fig. 15. Binding of NADH to HLADH-silica containing 21 (○) or 12 mg enzyme/g dry silica (●).

As discussed in section IV 3.3, the pH dependence for binding of NAD, ADP-ribose and AMP to HLADH also seems to be well conserved after immobilization.

Summarizing, the intrinsic properties of HLADH i.e. the activity, the coenzyme binding, the pH dependence and the K_m values for acetaldehyde/ethanol, seem to be well preserved after immobilization to tresyl chloride activated silica. As will be shown below, the porosity and the level of tresyl chloride activation of the silica are important factors that influence the intrinsic properties of the immobilized enzyme.

IV APPLICATIONS

1. Use of immobilization to study conformational changes in HLADH

Many enzymes are known to undergo conformational changes upon binding of substrates or effectors. It has been argued that immobilization of enzymes by multiple covalent linkages to a support would be a way to detect such changes. Trypsin, for example, has been coupled with and without its substrate resulting in different rates of inactivation by iodoacetamide. This was attributed to some "freezing" of the enzyme conformation (14). In addition, enzyme preparations that do not need soluble effectors for optimal activity might be obtained in this way.

From X-ray crystallographic studies, it has been found that HLADH undergoes a relatively large conformational change upon binding of coenzyme (160). The change involves a rotating motion of the catalytic domains relative to the coenzyme binding domains, resulting in that some residues shift positions by up to 7 Å relative to the apoenzyme. The structure of the coenzyme binding domain of each subunit is relatively unaffected upon binding. Thus, only small changes in the tertiary structure responsible for the binding of the AMP-portion of the coenzyme are observed (160).

HLADH (5 mg/g dry silica) was coupled in its ternary complex with NADH and isobutyramide to glycerylpropyl-silica (LiChrosorb Diol, Merck, 5 µm, 100 Å pores) highly activated with tresyl chloride (200 µmol tresyl groups/g dry silica) at pH 8.0, 20°C and overnight (135). After rigorous washing of the gel, in order to obtain the apoenzyme, an enzyme preparation (A) was obtained having lower dissociation constants (mean value: 50%) for NADH and NAD, when compared with a preparation (B) coupled together with ADP-ribose, which is known not to induce any conformational change (161). HLADH was also coupled to silica with a lower amount of tresyl groups (50 µmol/g) together with ADP-ribose (C) or NADH + isobutyramide (D). These preparations had dissociation constants similar to those obtained with preparation B above (K_d (NADH) about 0.5 µM). The binding of AMP, however, was virtually the same for all preparations, which might be expected (see above and ref. 160). The dissociation constant for AMP (at pH 7.5 about 120 µM) agreed well with values reported for the soluble enzyme (162). The dissociation constants were determined from Scatchard plots of bound radioactive nucleotide to the enzyme-gels. The coupling of enzyme was almost complete in all cases as judged from amino acid analysis.

The specific activity of preparation A was lower than for the other preparations, indicating that the dissociation rate of the coenzyme was lower in this preparation. The activities of preparations B-D were practically the same as that of the free enzyme.

In addition to the amount of tresyl groups, the pore size is also an important factor since 1000 Å pore silica did not give the effects described above. This is logical if one considers that a much larger portion of the surface of the HLADH molecule (with the approximate dimensions 40 Å x 55 Å x 100 Å (160) is expected to be surrounded by tresyl groups in the pores of 100 Å silica than in the large pore silica. The pores of the 100 Å silica are, in fact, only about 70 Å in diameter after coating with glycerylpropyl groups. That the enzyme actually was bound in the pores of 100 Å silica is substantiated by that the theoretical capacity of nonporous 10 µm spheres is only about 1 mg HLADH/g (70), i.e. five times less than the loading in preparations A-D.

The results obtained indicate that highly tresylated silica, at least to some extent, can preserve the ternary complex conformation of HLADH. It is to be expected that, in addition to the narrow pores that was used, the ability of tresylated supports to react with many types of nucleophilic groups on the enzyme (e.g. sulfhydryl, amino, guanidino, imidazole and tyrosinyl groups) was of importance in this experiment.

2. Peptide synthesis with immobilized chymotrypsin

There are many enzymatically catalyzed organic reactions of potential industrial interest where addition of organic solvents would be beneficial, either for increasing the solubility of substrates and/or for promoting product formation. To this end, both water-miscible and water-immiscible organic solvents have been employed (for reviews see refs. 163-165). High concentrations of organic solvents (up to 99%) have been successfully used in some water:water-immiscible organic solvent systems, the enzyme in the water-phase being protected from denaturation by the low solubility of organic solvent in water (166, 167) or by detergents creating reverse micelles (164). The partition coefficients of substrates and products between the phases are important for the outcome of the reaction in biphasic systems (163). For continuous operation with immobilized enzymes the risk of product precipitation in the carrier pores or in the water-phase, limit the application of biphasic systems.

The use of water-miscible organic solvents with soluble enzymes is limited by their often detrimental effect on enzyme solubility, activity and stability. The problem of aggregation is obviously solved by immobilizing the enzyme on a solid support. This has been taken advantage of for determination of kinetic constants of chymotrypsin bound to glass beads in up to 95% dioxane (12). It was found that while the acylation rate was largely unaffected, K_m increased drastically and the rate of deacylation decreased as expected from the decrease in water content with increasing cosolvent concentration. The increased K_m has been attributed to a simple hydrophobic effect; the substrate partitioning more favorable to the bulk solvent than to the active site, the higher the concentration of organic solvent (168). The increased K_m should however not constitute a problem in applications where high concentrations of substrates are possible. This is usually the case with the syntheses of short peptides by enzymes.

The interest in enzyme catalyzed peptide synthesis have greatly increased during the last few years because of an increasing awareness of the inherent advantages of utilizing enzymes in organic synthesis and by the large number of isolated proteases of different specificities. The sequence and stereospecificity of proteases, the minimal need for side chain protection and products free of racemisation are some of the advantages of enzymatic peptide synthesis compared with conventional chemical procedures (for reviews see refs. 169, 170).

Enzymatic peptide bond formation is effected by two major approaches (Fig. 16): (1) reversal of peptide bond hydrolysis, (equilibrium approach), e.g. the condensation of N-protected acyl component and C-protected amino component (protection is necessary with endopeptidases) and (2) aminolysis of ester substrates, (kinetic (nonequilibrium) approach), i.e. the enzyme reacts rapidly with the acyl component, which is in the form of an ester, to form the acyl-enzyme intermediate that reacts with the amino component or with water to form the desired peptide or the hydrolysed ester, respectively.

The condensation method has been used with all types of proteases, but is obligatory with acid or metalloproteases which do not form an acyl-enzyme intermediate. The method requires a high concentration of organic solvent, a large excess of the amine component or low solubility of the product (i.e. precipitation) to shift the equilibrium towards the peptide product side. Further, a large amount of enzyme is often

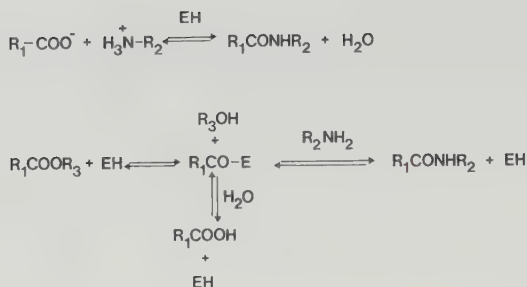


Fig. 16. Enzymatic peptide bond formation.

required to achieve a reasonable rate of condensation. The method has been successfully employed with both water-miscible (171, 172) and water-immiscible solvents (167).

The kinetic approach is favorable with thiol or serine proteases as they are acylated faster by ester substrates than by peptide products leading to product accumulation. The yield of peptide is obviously determined by the reactivity of the amine component, relative to that of water, towards the acyl-enzyme. For acyl-chymotrypsin, 1 M H-Ala-NH₂ is about 44 times more reactive than 55 M H₂O at pH 9.2 and 25 °C (173).

Numerous reports utilizing the kinetic approach for peptide syntheses both with soluble and insoluble enzymes can be found in the literature (169, 170). Data concerning the following parameters are however scarce or absent: (a) the influence of the concentration of different water-miscible organic solvents on peptide yield from ester substrate and on the secondary specificity and stability of the enzyme; (b) the influence of temperature on peptide yield and enzyme stability; (c) the influence of the level of support activation on the stability and secondary specificity of the immobilized enzyme. These parameters were studied in paper IV with α-chymotrypsin bound to tressylated agarose (Sephacrose CL-4B). Precipitation was avoided to take advantage of the reusability of the immobilized enzyme.

The yield of bound α -chymotrypsin (5 mg added/g wet gel) was 60, 68 and 78% when coupled to agarose with 225, 570 and 970 μ mol tresyl groups per g dry support, at pH 7.8, 4°C and overnight. The enzyme was coupled together with a competitive inhibitor (Ac-D-Trp-OMe) to minimize autolysis. The rate of peptide synthesis was good even in high concentration of organic solvents. The initial linear rate of product formation with 0.5 M Ac-Phe-OMe and 0.5 M H-Gly-NH₂ was actually higher in 50% DMF than in 10% DMF. The higher K_m in 50% DMF (cf. refs. 14,168) was compensated for by using higher concentration of substrates.

Most proteases exhibit a secondary specificity (169). For example, the saturation rate of chymotrypsin catalyzed hydrolysis of Ac-Phe-Ala-NH₂ is higher than for hydrolysis of Ac-Phe-Gly-NH₂. Thus, H-Ala-NH₂ has a higher reactivity than H-Gly-NH₂ towards the acyl-enzyme intermediate in the reverse reaction, i.e. peptide bond formation. Furthermore, chymotrypsin distinguishes between acceptor nucleophile enantiomers, the L-form being favored over the D-form.

Table 5. Chymotrypsin-agarose catalyzed peptide syntheses.

Solvent	Concentration (volume %)	Concentration of Ac-Phe-OMe (M)	Acceptor amine	Concentration (M)	Yield (%)
DMF	50	0.1	H-Ala-NH ₂	0.2	84
DMF	50	0.1	H-Ala-NH ₂	0.6	97
DMF	50	0.1	H-Gly-NH ₂	0.2	68
Acetone	50	0.1	H-Ala-NH ₂	0.2	84
Acetone	50	0.1	H-Gly-NH ₂	0.2	68
Glycerol	50	0.1	H-Gly-NH ₂	0.2	40
Butanediol	50	0.1	H-Gly-NH ₂	0.2	40
Butanediol	50	0.05	H-Ala-NH ₂	0.1	67
Butanediol	90	0.05	H-Ala-NH ₂	0.1	88
Butanediol	90	0.05	H-D-Ala-NH ₂	0.1	33
Butanediol	90	0.05	H-Gly-NH ₂	0.2	86

Chymotrypsin immobilized to either high or low activated agarose exhibits secondary specificity in up to at least 50% DMF and 90% 1,4-butanediol at room temperature (Table 5). Almost complete conversion of ester substrate to peptide could be effected by using an excess of the amine component in the aqueous-organic solvent mixture. With this immobilized enzyme system retrieval of the unreacted amine would be easy.

The yield of peptide increased with the concentration of organic solvent. The type of organic solvent was important, DMF and acetone gave higher yields than glycerol and 1,4-butanediol at the same volume percent. The latter type of solvents influenced the catalytic rate less than the first type and could be used at higher concentrations. The high viscosity and low ability of polyols to dissolve hydrophobic substrates makes them less suitable for continuous peptide syntheses with immobilized enzymes. It is well known that organic solvents of the polyol type show less detrimental effects on enzymes than dipolar aprotic solvents. This has been ascribed to "preferential hydration" of the protein surface in mixed aqueous-polyol solution (174). Dipolar aprotic solvents, on the other hand, have a greater tendency to bind to proteins (preferentially hydrophobic side chains) and thus may lower the concentration of water around the protein more effectively than the polyols. This may rationalize the finding in paper IV that the polyols had to be used at a much higher concentration than the dipolar aprotics in order to have a favorable effect on peptide yield.

Coupling of chymotrypsin to a higher activated support resulted in a preparation with higher stability (i.e. better conservation of initial synthetic activity) in aqueous-organic solvent mixtures compared with a preparation obtained from a lower activated support. The difference in stability increased with increased temperature, pH and DMF concentration. Thus, after 3 weeks in 50% DMF at 20°C there was twice as much activity left on the higher activated preparation than on the low activated support, while there was a ten times difference in activity after one day at 37°C. The increased stability obtained on coupling to the highly activated support can be explained by that this support allows more linkages to be formed with the enzyme, which decreases the tendency for unfolding of the enzyme. This is an analogy with previous reports on inactivation of immobilized enzymes by heat (50) or urea (53).

As water-miscible organic solvents are known to decrease the temperature of enzyme denaturation, enzyme stability in such solvents can be increased by lowering the temperature. This is well known from

cryoenzymology where water-miscible solvents are used in high concentrations at subzero temperatures (175, 176). In paper IV immobilized chymotrypsin was shown to be rapidly inactivated in 50% DMF at 37°C, but was quite stable at 4°C, 70% of the original synthetic activity remaining after 3 weeks.

As an additional advantage the yield of peptide increased considerably on lowering of the temperature. Thus, the yield of Ac-Phe-Gly-NH₂ was about 67% at 22°C, but 86% at -10°C.

The decreased solubility of substrates and products, as well as the decreased enzymatic activity with decreased temperature, limit the applicability of subzero temperatures for continuous synthesis by immobilized enzymes. However, as shown in paper IV, the rate of peptide synthesis at -10°C in 50% DMF, which was about 10 times lower than at room temperature, still allowed rapid conversions when ester substrate was employed. In addition, no precipitation was observed.

Thus the kinetic approach with immobilized chymotrypsin can be favorably accomplished in the presence of high concentrations of water-miscible organic solvents with high yields of peptide. The increased solubility of substrates and products in mixed aqueous-organic solvents makes the continuous use of the immobilized enzyme possible.

3. Affinity chromatography

The method used for immobilization of affinity ligands should be gentle so that the "ideal" characteristics of the support and the structural integrity of the affinity ligand are preserved as far as possible. The method should not create nonspecific adsorption effects and the linkages formed should be stable during chromatographic conditions. Especially for expensive ligands, it is important that the coupling is efficient. In some applications it is beneficial if the method allows for very high substitution of the support with immobilized ligand. In addition, it is advantageous if the method is easy to reproduce, giving predictable levels of activation. As discussed in section II 3 immobilization with organic sulfonyl chlorides seems to meet most of these requirements.

As was demonstrated above (cf. Table 3) a quite low level of support activation, i.e. 150-400 μmol tresyl groups per g dry agarose and 50-100 μmol tresyl groups per g of dry glycerylpropyl-silica (300 Å) is enough to obtain efficient coupling of biomolecules. This low activation level minimizes the risk of altering the properties of the proteins to be immobilized as well as the risk of interference from the sulfonyl groups remaining after coupling. It is usually sufficient to treat the support after coupling of protein with 0.2 M Tris-HCl buffer at pH 8 for 2 h at room temperature in order to remove any interference from remaining tresyl groups (135). Mercaptoethanol has also been used with biomolecules not sensitive to this compound (paper III). Affinity adsorbents prepared via tosyl chloride activation need a higher pH and a higher concentration of Tris or mercaptoethanol and, in cases of very high activation levels, an increased temperature for removal of interfering active groups after coupling (I,II, and ref. 142).

Many of the affinity experiments that are described in this thesis were made on adsorbents prepared from highly activated supports. Despite this, nonspecific effects or adverse effects on the affinity properties of the immobilized ligands were not detected after treatment with Tris or mercaptoethanol.

Some examples of affinity chromatography with affinity adsorbents prepared via sulfonyl chloride activation are given below (I, III, V and ref. 142). In addition to these examples, the specific binding of yeast and bacterial cells to proteins (concanavalin A and IgG) immobilized on tresyl chloride activated silicon wafers has recently been demonstrated (141). The binding of protein to the wafers was followed with ellipsometry. It was shown that smooth monolayers of covalently bound protein could be formed on the tresyl-wafers.

3.1. Agarose bound ligands

a) 5'-AMP and 2',5'-ADP analogues (I,III)

About one fifth of the 2122 enzymes given a name are NAD^+ - or NADP^+ -dependent dehydrogenases (177). Many dehydrogenases contain a nucleotide-binding fold that recognize the AMP-portion of the coenzyme. Many other enzymes that utilize AMP, ATP or other adenine nucleotides as substrates or effectors contain a similar domain. Thus, immobilized 5'-AMP derivatives have been useful for the purification of a number of dehydrogenases, kinases and other enzymes (17,18,178). Analogues based

on 2',5'-ADP have been employed for the purification of NADP⁺-dependent enzymes. This type of affinity chromatography, where a given type of group specific ligand is utilized for the purification of a number of biomolecules, has been termed general ligand affinity chromatography (179). Development of group specific adsorbents based on adenine nucleotides are not only of potential interest for preparative purposes, but also for clinical analysis, where a number of tests are based on the presence of dehydrogenases and kinases.

The adenine nucleotide ligands can be directly attached to the support, but as with other low molecular weight affinity ligands, it is usually favorable to attach the ligand to the support via an extension arm (a "spacer"). The main functions of the spacer is to make the immobilized ligand sterically available to the macromolecule and to increase the flexibility of the ligand, facilitating correct orientation for binding. The optimal length of the spacermolecule, especially for hydrophobic spacers, is usually 8-10 Å, which corresponds to the length of the extended 1,6-diaminohexane spacer (180).

The spacer is attached at a position that is thought to be not vital for the biospecific interaction between the ligand and enzyme. Spacers have been attached to AMP at N⁶- or C⁸-positions of the adenine ring and at the ribose ring (178). The N⁶-derivatives are often preferred for the purification of dehydrogenases, while the C⁸-derivatives have been advocated to be favorable in many cases for the purification of kinases (181,182). As to the influence of different spacers on the affinity between the soluble ligand and the enzyme see paper V and section IV 3.2.

The attachment of the ligand to the spacer is done in two principal ways: the preassembly approach and the solid phase synthesis approach (178). In the former, which is to be preferred for construction of adsorbents with minimal nonspecific adsorption, the spacer is attached to the adenine nucleotide prior to the immobilization. In the solid phase approach, the spacer is first immobilized to the support, before attachment of the ligand. This is a practically simpler method, but inevitably leads to side reactions and may cause nonspecific adsorption by unmodified spacer molecules.

Since affinity adsorbents based on adenine nucleotides bind a large number of different enzymes, the purification is dependent on the specificity of the elution step. Elution with enzyme-specific counter ligands (biospecific elution) is therefore usually preferred over less

specific elution methods. For dehydrogenases, biospecific elution by ternary complex formation, as exemplified in paper II and III is a useful principle to obtain additional resolving power. Covalent adducts between the coenzyme and the other enzyme substrate have been found even more efficient (183). For a comprehensive review on general ligand affinity chromatography with adenine nucleotides see ref. 178.

It is known that the N^6 -(6-aminoethyl) analogues of 5'-AMP (part of the NAD^+ molecule) and 2',5'-ADP (part of the $NADP^+$ -molecule) can discriminate between NAD^+ -dependent and $NADP^+$ -dependent enzymes (184). Paper I shows that these analogues after coupling to tosyl-agarose exhibited similar affinity characteristics as when coupled to CNBr-activated agarose. Thus, NAD^+ -dependent lactate dehydrogenase and $NADP^+$ -dependent 6-phosphogluconate dehydrogenase bound specifically to gels with 5'-AMP and 2',5'-ADP analogues, respectively. No irreversible adsorption of the enzyme was found, as virtually all of the bound enzyme was recovered by biospecific elution with NADH or $NADP^+$. Furthermore, beef heart lactate dehydrogenase was purified in a single step on a column with immobilized 5'-AMP analogue. Bound enzyme was eluted biospecifically by ternary complex formation with NAD^+ and pyrazole. The binding capacity for lactate dehydrogenase of the immobilized 5'-AMP analogue under column conditions was 2.8 mg per g of settled gel, which is similar to values obtained under similar conditions for CNBr coupled ligand (185). Later studies with the ligands bound to tresyl-agarose revealed the same behavior as above (unpublished observations).

b) Soybean trypsin inhibitor (I,III)

This protein is an example of a general ligand with high molecular weight (mol wt 21 500). It has been used in affinity chromatography mainly for removal of contaminants (peptides, proteases) from commercially obtained trypsin preparations (17). The immobilized inhibitor binds active trypsin and chymotrypsin at neutral pH. Separation of the bound enzymes is accomplished by a decreasing pH-gradient or by biospecific elution with competitive inhibitors.

In paper I and III it was found that STI, when bound to tosyl- or tresyl-agarose, showed intact affinity properties similar to those reported when the inhibitor was bound to CNBr- or bisoxirane-activated agarose. The obtained capacity of the tresyl coupled inhibitor (11 mg per g of settled gel) for trypsin (9 mg per g of settled gel) is also

similar to the previously reported capacities of the immobilized inhibitor (98). In this context it may be mentioned that trypsin, chymotrypsin and albumin did not interact in either 0.025, 0.1 or 1.0 M phosphate buffer with highly tresyl chloride activated agarose, that had been treated with 0.1 M Tris-HCl (pH 8.0) for 5 h at 20°C. Similar experiments were carried out with tosyl chloride activated agarose (paper I).

c) Concanavalin A (paper III)

This protein belongs to a group of proteins which bind sugar and are isolated mainly from plant sources, i.e. the lectins. Concanavalin A binds preferentially α -D-glucopyranosyl and α -D-mannopyranosyl residues and recognizes carbohydrate containing biomolecules that have these structures on their surface. Concanavalin A and other lectins immobilized to agarose have been used for purification of glycoproteins, polysaccharides, viruses, detergent solubilized membrane components and even whole cells (17,18,186). Immobilized Concanavalin A has also been used in analysis of carbohydrate containing compounds (187).

As mentioned above (Table 3) concanavalin A was found to bind very efficiently (97% yield) to tresyl chloride activated agarose. The immobilized lectin was used for the purification of commercially obtained horse radish peroxidase. The biospecifically eluted peroxidase was considered pure from the obtained A_{403}/A_{280} ratio. Concanavalin A has also been immobilized to tresyl chloride activated glyceryl-propyl-silica in high yields at low pH (6.0) (188). The immobilized protein was integrated with HPLC equipment and used for the rapid separation of glycosides and glycoproteins.

d) Protein A and immunoglobulins

The properties of Protein A (142) and rabbit IgG (189) coupled to sulfonyl chloride activated supports have also been studied. On application of human serum IgG to a column with immobilized protein A at pH 7 a small fraction, probably representing IgG of subclass 3 that is known not to bind to protein A, passed unretarded. Virtually complete elution of bound IgG was effected by lowering the pH to 2.2 with 0.2 M glycine-

-HCl. The binding capacity for IgG was very high (about 70 mg per g of settled gel containing 4.5 mg protein A). The gels were used repeatedly for 4 months at 4°C without any decrease in capacity or alteration in affinity properties.

Nustad et al. coupled sheep anti-rabbit IgG to hydroxyl group containing polymethylmethacrylate particles (Dynosphers XP-4001) activated with either tresyl chloride, tosyl chloride or carbonyldiimidazole (189). About the same yield of bound IgG was obtained when coupled at pH 7 to the tresyl-polymer as when coupled at pH 9.5 to the tosyl- or the carbonylimidazole-polymer. The binding capacity for rabbit IgG approached the theoretical for the preparations obtained using the sulfonyl chlorides. Antibodies coupled to the carbonyldiimidazole-activated polymer, however, had a 50% lower binding capacity than the theoretical. Nonspecific binding was not observed and the preparations showed no decrease in capacity over a time period of several months.

3.2. Glycerylpropyl-silica bound ligands

High performance liquid affinity chromatography (HPLAC), the combination of HPLC equipment with affinity chromatographic principles, is characterized by its high selectivity and rapid separations (70,123, 190). The technique is expected to be useful in clinical analysis and possibly also for isolation of sensitive biomolecules. It can also be advantageous for concentration of a group of compounds, which subsequently are analyzed on an ordinary HPLC column (191). In paper V, the potential of HPLAC for rapid screening of equilibrium and rate constants of immobilized enzymes is discussed.

In order to obtain optimal separations, smaller and more uniform particles are used than in ordinary affinity chromatography. As a consequence of this higher pressure falls are created and rigid supports are needed. Glycerylpropyl-coated macroporous silica conforms to these requirements and, in addition, it can easily be derivatized with reactive groups for coupling of affinity ligands. At present, it seems to be the best choice of support for HPLAC, despite its relatively low chemical stability at alkaline pH and the slight nonspecific adsorption of some proteins.

The methods that have been employed for coupling of affinity ligands to glycerylpropyl-silica are: (1) direct coupling via the oxirane groups introduced after coating of silica with γ -glycidoxypropylsilane (on acidic hydrolysis of this oxirane coating, glycerylpropyl-coated

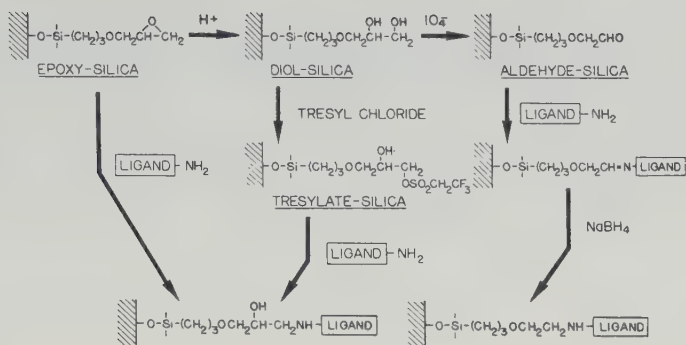


Fig. 17. Coupling of ligands to silica.

silica is obtained); (2) periodate oxidation of the 1,2-dihydroxy groups on glycerylpropyl-silica and subsequent coupling of amino containing ligand via Schiff's base formation and reduction with NaBH_3CN or NaBH_4 ; (3) activation of the diol groups with tresyl chloride (Fig. 17).

For coupling of aromatic amines the oxirane method seems to be sufficiently rapid to give substantial yields of bound ligand (192). The method has, however, very low efficiency for coupling of aliphatic amines or proteins at the near neutral pH that has to be used with the alkaline pH sensitive silica (188). Further, to remove unreacted epoxide groups, high concentrations of mercaptoethanol has to be used, which is incompatible with many proteins (70).

The periodate method is efficient for coupling of amino group containing ligands even below pH 7 (70,72). The reduction step can, however, be harmful to sensitive biomolecules (75,76).

Glycerylpropyl-silica is supplied dry which makes the activation step with sulfonyl chlorides very simple and easy to reproduce, as no intermediate washing steps for the activation in acetone is needed (paper III and V). After the activation and washing of the support, it can be freeze-dried, without any special precautions (as is necessary with agarose), and stored until required. The coupling of ligands is very efficient at near neutral pH (see Table 3). As an example, high amounts of concanavalin A was bound to tresyl-silica and in high yield even below neutral pH (6.0) (188).

Tosyl chloride activated silica is less suitable as it requires a more alkaline pH for coupling which may be harmful to this support. Tosylated silica should, however, be useful for immobilization of ligands soluble in organic solvents (see paper II and section III 3). The possibility to couple ligands containing thiol groups and thus obtain electroneutral, stable S-C linkages with the support constitutes an important advantage over the periodate method.

Some properties of N^6 -(6-aminohexyl)-5'-AMP and HLADH, when coupled to tosyl chloride activated glycerylpropyl-silica and used as adsorbents in HPLAC, are discussed below.

a) N^6 -(6-aminohexyl)-5'-AMP (paper III)

This ligand was coupled overnight at pH 7.5 and 20°C to glycerylpropyl-

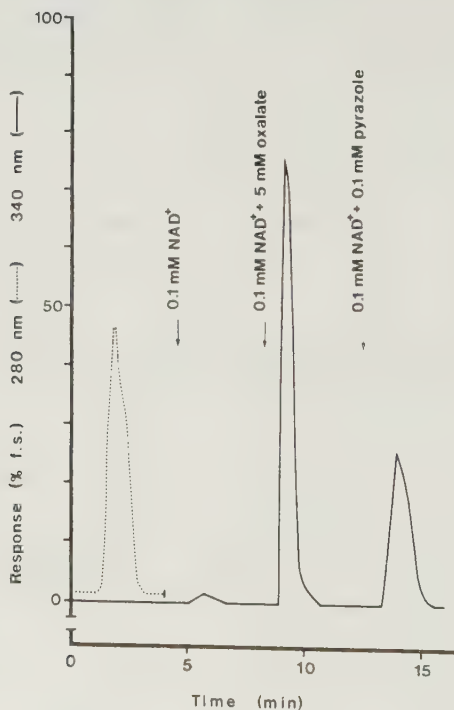


Fig. 18. Separation of bovine serum albumin, beef heart lactate dehydrogenase and horse liver alcohol dehydrogenase on an AMP-silica column.

silica (100 Å pores) activated to saturation with tresyl chloride. The yield of bound ligand was about 80% with 30 µmol AMP analogue bound per g of dry support. The support was treated with 0.1 M mercaptoethanol for 4 h at 20°C and pH 7.5, to remove unreacted tresyl groups. The AMP-silica was packed in an HPLC column and its properties tested by application of a mixture of two dehydrogenases, i.e. HLADH and LDH, and a protein lacking affinity for the AMP analogue, bovine serum albumin. In Figure 18 is shown the elution pattern obtained when biospecific elution of the bound dehydrogenases were effected by a short pulse of the ternary complex forming compounds. The separation was effected very rapidly, i.e. in 15 minutes. The albumin did not interact with the adsorbent as it passed unretarded through the column, whereas the dehydrogenases were completely retained. The biospecific nature of their interaction with the support is indicated by the selective elution of LDH and HLADH that was achieved by low concentrations of NAD^+ and oxalate or NAD^+ and pyrazole, respectively. The practically complete recovery of injected proteins and the relatively narrow peaks without "tailing" are indicative of a minimum of nonspecific interactions with the support and of a complete quenching of remaining tresyl groups despite the exceedingly high activation level.

b) Horse liver alcohol dehydrogenase (paper V)

In paper V an immobilized enzyme (HLADH) was utilized as a general ligand for the purification of low molecular weight inhibitors and substrates. The first use of an immobilized enzyme for purification of biomolecules was reported in 1966 when immobilized trypsin and chymotrypsin were used for purification of pancreatic inhibitors (193). Since then very few reports using this approach have appeared, probably because the adsorbents, in many cases, would be quite unstable and expensive, having a relatively low binding capacity compared with conventional purification procedures for small biomolecules. In an example showing the potential of immobilized enzymes for purification of complex isomeric compounds, difficult to separate by conventional chromatographic techniques, immobilized apoflavodoxin was utilized to purify commercially obtained riboflavin 5'-phosphate from riboflavin 4'-phosphate and other impurities (194). Recently, silica-bound albumin has been integrated with HPLC and utilized for the analytical resolution of a number of racemic compounds. High enantiomeric separation factors were obtained (195).

Furthermore, affinity chromatography utilizing immobilized enzymes would be a way to detect and purify new chemotherapeutic agents interacting with enzymes.

By immobilizing the enzyme to small uniform silica particles and integrating the support with HPLC equipment as in paper V, the number of separations that can be performed in a given time should be considerable increased compared with having the enzyme bound to larger, soft beads. The amount of compound needed for injection would also be lower because of the sharper peaks obtained with the smaller, more uniform particles. The use of this type of system for the rapid screening of K_d for retained material, was demonstrated in paper V (see next section).

HLADH was bound to tresylated glycerylpropyl-silica with practically complete retention of activity and coenzyme binding sites as judged from activity and equilibrium measurements (Table 4 and section III 4). In Figure 19 is shown the separation of a mixture of AMP, ADP and ADP-ribose on HLADH-silica. As expected from what is known of the interactions with the soluble enzyme, ADP is least retained, and ADP-ribose is last eluted on the column.

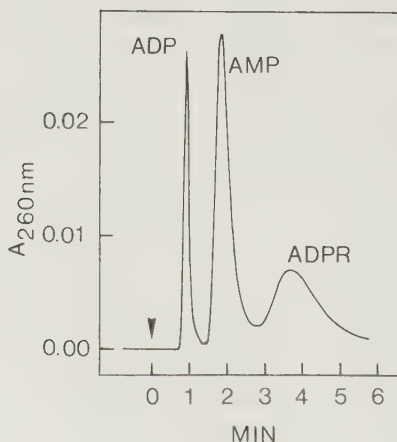


Fig. 19. Separation of adenine nucleotides on HLADH-silica.

3.3. Quantitative affinity chromatography with immobilized HLADH

a) Determination of equilibrium constants

The use of conventional affinity chromatography to quantitate dissociation constants between soluble or immobilized ligands and soluble enzymes has been amply documented (196). Equations have been derived that enables determination of equilibrium constants from either zonal (197-199) or frontal experiments (200). A drawback with the frontal method compared with the zonal is that larger amounts of soluble enzyme is required. Further, the precision of the methods are expected to be similar, provided the concentration of the soluble enzyme is kept considerable lower in the column than that of the immobilized ligand in the zonal experiment (196, 200). A zonal method based on gradient elution with competing soluble ligand has also been reported (201).

In paper V, an equation allowing the calculation of dissociation constants (K_d) from zonal experiments with immobilized enzyme and soluble ligand was obtained analogously to that previously derived for the reverse case, i.e. immobilized ligand and soluble enzyme (197-200). An equation permitting evaluation of the dissociation constant of a competitively eluted ligand was similarly obtained. Thus, in the case of competitive elution with a counter ligand C:

$$K_d = \frac{(\text{HLADH})}{k'} \cdot \frac{K_d(C)}{K_d(C) + (C)} \quad (1)$$

where (HLADH) is the total concentration of HLADH binding sites in the liquid phase volume of the column and $K_d(C)$ is the dissociation constant of the counter ligand. This expression simplifies to

$$K_d = \frac{(\text{HLADH})}{k'} \quad (2)$$

in the absence of a competitive ligand. The mass distribution ratio, k' , was calculated from the elution times of the adsorbed, T_e , and nonadsorbed compounds, T_o , respectively, using the formula (122):

$$k' = (T_e - T_o) / T_o \quad (3)$$

Using these equations, K_d could be rapidly evaluated for a number of compounds known to bind to the nucleotide binding site of the soluble

enzyme. The correlation between the dissociation constants calculated from the experimental data and the literature values for soluble HLADH complexes was good (Table 6). The dissociation constant obtained for AMP was almost the same as that determined on HLADH-silica by equilibrium measurements (cf. p 43).

Table 6. Determination of dissociation constants for adenosine and various adenine nucleotides by chromatography on HLADH-silica.

Compound	K_d (μM)	
	Calculated	Literature
Adenosine	1150	3700
AMP	134	70
N^6 -(6-aminohexyl)-5'-AMP	51	32
N^6 -(2-aminoethyl)-5'-AMP	1030	
N^6 -carboxymethyl-5'-AMP	107	
ATP	5000	
ADP-ribose	38	18
NAD	120	96

NADH was bound very strongly to the HLADH-silica column because of the low dissociation constant of the enzyme-NADH complex. NADH was however, eluted in the presence of a competitive ligand in the eluant. Using equation 1 excellent agreement with previous equilibrium measurement on HLADH-silica ($K_d = 0.8 \mu\text{M}$ for NADH; see section III 4) was obtained with NAD^+ or AMP as counter ligands (K_d for NADH is 1.0 or $0.6 \mu\text{M}$, respectively).

Furthermore, the effect of pH on the retention of NAD and ADP-ribose on HLADH-silica was as expected from the pH dependence of the soluble enzyme. Consequently, the variation of the calculated dissociation constants with pH was similar to that reported for the soluble enzyme.

The spacer arm is known to affect the binding between enzyme and affinity ligand and consequently the behavior of enzymes on affinity columns. Thus, the 1,6-diaminohexane spacer attached at the N^6 -position of the adenine ring is thought to increase the binding

strength of many dehydrogenases to immobilized 5'-AMP (178). This has been attributed to a hydrophobic interaction between the spacer and the hydrophobic groups near the adenine binding site when the AMP-part of the ligand is biospecifically bound (198). This phenomenon, termed compound affinity, is distinguished from the "true" nonspecific interactions that might occur between the spacer by itself and the enzyme. On application of a number of adenine-, AMP-, and NAD-analogues of potential utility as general ligands, a large variation in retention on HLADH-silica was observed with different spacers. The difference was enough pronounced to allow separation of a mixture of AMP-analogues on a HLADH-silica column.

The K_d -value obtained for N^6 -(6-aminohexyl)-5'-AMP was less than half of that of 5'-AMP and correlated well with the literature data for the soluble enzyme (Table 6). Interesting was the very large difference in retention for the AMP-analogues substituted at the N^6 position with carboxymethyl or 2-aminoethyl groups. Thus, the binding of the 2-aminoethyl derivative was about 10 times weaker than the binding of the carboxymethyl derivative. Assuming that the effect was caused by compound affinity, it could be attributed to an electrostatic interaction of the aminoethyl or the carboxymethyl derivative with a positively charged arginine side chain in HLADH (arg₂₇₁). This residue is situated close to the hydrophobic adenine binding site, partially shielding this site from the solvent (160).

The range of dissociation constants that can be measured with the above technique seems quite broad. With the columns used in paper V, containing 150-300 μ M HLADH-subunits, dissociation constants in the range 0.6-200 μ M could be determined with reasonable accuracy. For compounds with higher dissociation constants, the enzyme loading may be increased. To facilitate this, 300 Å pore silica, which has a higher surface area than the 1000 Å silica used in paper V, might be employed. The lower limit of dissociation constants that can be measured with this chromatographic technique is set by the dissociation rate constant of the ligand. If this constant is too small, elution of the ligand, even at very low concentration of immobilized enzyme, would not occur within a feasible amount of time. Counter ligands will have no effect at this point since the dissociation step is unimolecular. In cases where the association rate constant is diffusion limited, a practical lower limit of $K_d = 10^{-9}$ M can be estimated in analogy with previous estimates for the reverse situation, i.e. immobilized ligand (70,196).

The observations made in paper V clearly show that zonal affinity chromatography can be favorably used to estimate equilibrium constants for the interaction between the immobilized enzyme and the soluble ligand.

The main advantages with this "reverse" situation for quantitative affinity chromatography are : (1) very small amounts of valuable ligands are needed; (2) no elaborate synthesis of immobilized affinity ligands, that act competitively with the compound for which K_d is to be determined, is required; (3) the actual amount of immobilized binding sites interacting with the soluble compound is easier to estimate than when a low molecular weight ligand is immobilized; (4) the combination with HPLC-technique makes it a very rapid method for screening of dissociation constants of substrates, inhibitors and effectors as well as of potential chemotherapeutic agents and affinity ligands interacting with the enzyme. In addition, subunit interactions of multimeric enzymes and enzyme-enzyme interactions should be possible to quantitate by this type of HPLC-technique.

The general utility of the method described in paper V with other enzymes is limited by the fact that relatively large amounts of enzyme is required and by the fact that the enzyme's binding characteristics can be changed after immobilization. This means that the immobilization method should be as efficient and mild as possible. In paper V it was shown that the tresyl chloride method fulfilled these requirements as virtually 100 % of the enzyme added was coupled and with retained binding characteristics. The use of "minicolumns" for HPLAC (202) would minimize the amount of enzyme that is needed. The conventional zonal procedure for K_d determination with immobilized ligands, will probably benefit by introduction of the HPLC-technique, as the amount of soluble components (enzyme and ligand) and time can be minimized.

b) Determination of rate constants

For optimal separation of compounds by affinity chromatography peak widths must be minimized. This is also an important factor in HPLAC, especially for the optimal resolution of compounds showing a small difference in retention volumes. A convenient measure of the performance of a column is the experimental plate height, H_{exp} , which is calculated from the column length, L , the elution volume, V_e , and the peak base width, DV_e , using the relation (122):

$$H_{exp} = L/16(V_e/DV_e)^2$$

In HPLAC the contributions in the column to the plate height can be divided in two major categories. The first is due to dispersion and diffusion effects (H_d) and the second is due to the kinetics of the solute-immobilized ligand interaction (H_{kin}) (70). The kinetic contribution at a given flow rate becomes increasingly important the slower the dissociation rate of the solute-ligand complex and the smaller and more uniform the particles (203).

Provided H_{kin} can be evaluated and equations relating it to the dissociation rates of the solute-ligand interactions are available, calculation of kinetic constants from experimental HPLAC data would be possible. Such equations, relating H_{kin} to dissociation rates have been proposed by several authors (203-206). Their application to systems with low molecular weight ligands immobilized on large, nonuniform agarose beads and where the diffusing molecule is a large molecular weight enzyme, have been unsuccessful. With this type of system the obtained rate constants were several orders of magnitude lower than solution data (197). This is most probably because H_{kin} is negligible compared with H_d in such systems. More accurate determinations of H_{kin} would be possible using the reverse situation, with the enzyme immobilized and the low molecular weight ligand diffusing. Small uniform beads, as in the case of HLADH-silica, also decrease H_d (122).

In paper V different equations for determination of rate constants from elution profiles were used, but the obtained values were about 2 orders of magnitude lower than those expected from kinetic measurements in solution. Probably even with this chromatographic system the kinetic contribution to the plate height was too small to allow its correct determination.

To minimize the diffusion limitations further, the enzyme should be immobilized on the surface of compact silica beads. Such particles should be small (about $1\ \mu\text{m}$) to allow a proper loading. Even with this system precautions have to be made. Extra column bandspreading has to be correctly compensated for, the contribution to the band broadening from an eventual subpopulation of sterically hindered enzyme binding sites has to be evaluated and the effect of enzyme loading on the diffusion resistance at the particle surface has to be considered (205).

V CONCLUSION

The present study shows that organic sulfonyl chlorides are suitable reagents for mediating covalent immobilization of biomolecules to various supports carrying hydroxyl groups. The activation step is simple and is effected in a short time period. The degree of activation is easy to predict and reproduce and the activated gel does not lose coupling capacity when stored for several weeks in aqueous media. The sulfonyl chloride method can also be utilized for activation of water-soluble polymers, such as polyethyleneglycol (PEG), which are useful in biochemical separations and for enzyme modification.

Supports activated with tresyl chloride can be used for coupling of pH-sensitive biomolecules, since high yields are obtained at neutral pH. In the pH-range 9-10.5, tosylated supports are effective for coupling of ligands that are stable in this range.

Enzymes were bound in high yields and with high retained activities to tresylated supports. Thus, horse liver alcohol dehydrogenase (HLADH) was coupled in almost 100 % yield to glycerylpropyl-silica. The enzyme was immobilized with practically complete retention of specific activity and of coenzyme binding site properties.

The many types of nucleophiles (e.g. sulfhydryl, amino and aromatic hydroxyl groups) that can react with the sulfonate esters are important when many linkages between the protein and the support is desired, e.g. for stabilization or "freezing" of the protein conformation. Furthermore, one is not restricted to spacers containing amino groups when constructing affinity ligands. By using thiol spacers, uncharged S-C bonds are obtained.

Affinity ligands, bound to either tosyl chloride or tresyl chloride activated agarose were shown to retain their affinity properties. The tresyl chloride method is ideally suited for immobilization of affinity ligands to glycerylpropyl-silica which can be used in high performance liquid affinity chromatography. This has been demonstrated by the quantitative immobilizations at near neutral pH of N⁶-(6-aminohexyl)-5'-AMP, concanavalin A and HLADH and the subsequent application of the adsorbents in HPLAC.

Zonal HPLAC was utilized for rapidly estimating the dissociation constants between the immobilized enzyme and the soluble ligand. This method should be valuable in the study of enzymes, allowing rapid screening of new inhibitors and in discovering ternary complexes in

which the enzyme is involved. Attempts to use this chromatographic technique for the quantitation of association/dissociation rate constants of the enzyme-ligand complex were, however, largely unsuccessful. The use of compact and even smaller beads than those used here for immobilization of the enzyme might however, lead to a better quantitation. This technique may then be a good complement to the different methods which are presently available for determination of rate constants.

Chymotrypsin immobilized to tresylated agarose was found to be useful for peptide syntheses, promoting high yields from ester substrates in water-organic solvent mixtures. The secondary specificity of the enzyme seemed to be conserved after immobilization and in high concentration of water-miscible organic cosolvents. The use of subzero temperatures, which were found to increase peptide yield from ester substrates and to increase enzyme stability in aqueous-organic solvent mixtures, may prove useful in peptide syntheses with immobilized thiol or serine proteases.

The applications of covalent immobilization methods and immobilized biomolecules for analytical, clinical and technological purposes and for fundamental studies are increasing. It may be expected that the here described organic sulfonyl chlorides may fulfill the requirements in most of these applications. One of the great advantages with this immobilization procedure is that a variety of sulfonyl chloride derivatives exists, which gives the sulfonates a broad reactivity spectrum. This makes it likely that one will be able to find a suitable derivative for the particular application in mind.

VII ACKNOWLEDGEMENTS

I want to express my deepest gratitude to Professor Klaus Mosbach for his encouraging support of my work and for valuable discussions.

I also want to thank Dr. Per-Olof Larsson for fruitful cooperation and advice.

Many thanks are also due to all my colleagues at the Pure & Applied Biochemistry Department, Lund, for creating a stimulating working atmosphere and for valuable discussions.

Finally, I want to thank Dr. Staffan Birnbaum for revising the English of this manuscript and Mrs Inge Cissé for typing the manuscript.

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p-Toluenesulfonyl Chloride as an Activating Agent of Agarose for the Preparation of Immobilized Affinity Ligands and Proteins

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(Received April 14/August 11, 1980)

A number of biomolecules were coupled covalently by nucleophilic displacement to agarose preparations substituted with tosyl groups. In one series of experiments *N*⁶-(6-aminohexyl)-adenosine 5'-monophosphate and *N*⁶-(6-aminohexyl)adenosine 2',5'-bisphosphate were bound by their terminal amino groups to the polysaccharide support. It could be shown that from a mixture of lactate and 6-phosphogluconate dehydrogenase the immobilized monophosphate showed bio-affinity only for NAD⁺-dependent lactate dehydrogenase, whereas the immobilized bisphosphate showed affinity only for the NADP⁺-dependent 6-phosphogluconate dehydrogenase. Furthermore, the immobilized monophosphate (5 μmol/g wet gel) was applied for the single-step purification of lactate dehydrogenase from crude beef heart extract.

To demonstrate the immobilization of proteins, soybean trypsin inhibitor (75 mg/g dry support) was immobilized to tosylated agarose, tested as affinity chromatography material and shown to bind 60 mg trypsin/g dry gel. Horseradish peroxidase and horse liver alcohol dehydrogenase were used as model enzymes. Although no optimization had been attempted, the former (approximately 70 mg/g dry support) had a coupling yield of approximately 18% with a specific activity (relative to soluble enzyme) of approximately 10%, whereas approximately 60% of alcohol dehydrogenase was coupled (approximately 100 mg/g dry support) with a specific activity of approximately 25%.

A number of immobilization techniques leading to covalent attachment of biomolecules, such as affinity ligands [1] or enzymes [2], are known and have been applied. Probably the most common procedure for binding to polysaccharide supports involves activation by cyanogen bromide [3], while another frequently used polysaccharide support carries epoxy groups [4]. Although these procedures have proved successful, they can have drawbacks under certain conditions.

We therefore feel that the more coupling methods that are available the better the chances of finding an immobilization procedure tailor-made for a particular purpose. We have found tosylated polysaccharides to be suitable alternative supports for immobilization. It is known from the chemistry of soluble saccharides

that reaction of hydroxyl groups with *p*-toluenesulfonyl chloride (tosyl chloride), forms corresponding esters (tosylates) which have excellent leaving properties in reactions with nucleophiles giving stable linkages between ligand and saccharide carbon [5,6]. This finding prompted us to try to develop a procedure suitable for the binding of biomolecules to polysaccharides. The mechanisms of these reactions are analogous to those of soluble saccharides [5,6] and are thought to involve the steps shown in Scheme 1.

The present paper describes the coupling of various biomolecules including coenzymes and proteins to these supports, and demonstrates their use in general ligand affinity chromatography [7] as well as enzyme immobilization.

MATERIALS AND METHODS

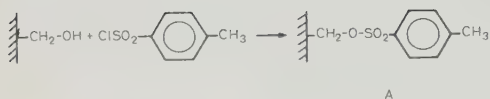
Materials

Dried dioxane (max. 0.01% H₂O) was bought from Merck (Darmstadt, FRG); *p*-toluenesulfonyl chloride (98%) was from Aldrich-Europe (Beersee,

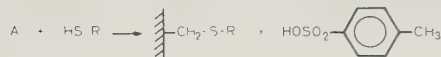
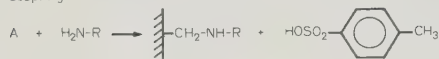
Abbreviations. Tosyl, *p*-toluenesulfonyl; Ahx⁶Ado5'*P*, *N*⁶-(6-aminohexyl)-adenosine 5'-monophosphate; Ahx⁶Ado(2',5')P₂, *N*⁶-(6-aminohexyl)-adenosine 2',5'-bisphosphate; BzArgOEt, *z*-*N*-benzoyl-L-arginine ethyl ester.

Enzymes. Alcohol dehydrogenase (EC 1.1.1.1); lactate dehydrogenase (EC 1.1.1.27); 6-phosphogluconate dehydrogenase (EC 1.1.1.44); peroxidase (EC 1.1.1.7); trypsin (EC 3.4.21.4).

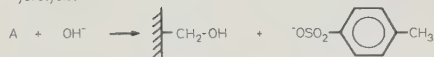
Activation:



Coupling:



Hydrolysis:



Scheme 1. Reactions involved in the binding of biomolecules to polysaccharides using tosyl chloride

Belgium). Sepharose CL-6B was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). *N*⁶-(6-Aminoethyl)-adenosine 5'-monophosphate (Ahh⁶Ado5'*P*) and *N*⁶-(6-aminoethyl)-adenosine 2',5'-bisphosphate [Ahh⁶Ado(2',5')*P*₂] were kindly provided by Dr P.-O. Larsson from our institute (these chemicals are commercially available from Sigma Chemicals, St Louis, MO, USA, for description of synthesis see also ref. [14]). Lactic dehydrogenase (beef heart, type III, 460 units/mg), 6-phosphogluconate dehydrogenase (yeast, type IV, 37 units/mg), trypsin inhibitor (soybean, type I-S), peroxidase (horseradish, type II, 165 purpurugallin units/mg), trypsin (bovine pancreas, type III, 11 700 BzArgOEt units/mg), albumin (bovine, fraction V), alcohol dehydrogenase (horse liver, 1.85 units/mg), pyruvate (sodium salt, type II), β-NAD⁺ (yeast, Sigma grade), β-NADP⁺ (yeast, Sigma grade), 6-phosphogluconic acid (tri-sodium salt, grade IV), α-*N*-benzoyl-L-arginine ethyl ester · HCl (BzArgOEt) and 4-amino-antipyrine were purchased from Sigma Chemicals (St Louis, MO, USA). Hydrogen peroxide (30%) was obtained from BDH Chemicals Ltd (Poole, UK). All other chemicals were of analytical grade and used as supplied.

Preparation of Tosylated Agarose

Wet Sepharose CL-6B was washed with 3 × 10 gel volumes of water, water:dioxane (3:1, v/v), water:dioxane (1:3), dioxane and finally with dried dioxane (containing less than 0.01% water). The purpose of

this washing procedure was to get rid of water, which may hydrolyze the tosylating agent, tosyl chloride.

7 g wet weight of the above Sepharose was then transferred to a round-bottomed flask containing 1 g tosyl chloride dissolved in 2 ml dried dioxane. During magnetic stirring 1 ml pyridine was added dropwise for about 1 min. After a 1-h reaction at room temperature, the gel was washed with 2 × 10 gel volumes dioxane and then gradually transferred back to water by reversing the washing scheme described above. The gels were stored in distilled water at 4 °C until used.

The amount of bound tosyl groups was 1.15 mmol/g dry weight corresponding to 0.43 tosyl group per disaccharide unit (*M*_r, 306). This figure was derived from the amount of sulphur determined by elemental analysis and from the ultraviolet spectrum of the tosylated gel (λ_{max} = 261 nm). The gels prepared in the above way were used for the coupling for ligands.

Coupling of Affinity Ligands

Ahh⁶Ado5'*P* and Ahh⁶Ado(2',5')*P*₂ were coupled to the tosylated gel according to the following procedure. 0.7 g wet tosylated agarose, washed with 0.5 M NaHCO₃ (pH 10.7), was added to a solution of 20 mg Ahh⁶Ado5'*P* or Ahh⁶Ado(2',5')*P*₂ in 0.3 ml 0.5 M NaHCO₃ (pH 10.7). Coupling was carried out at 40 °C in a water bath with a shaker for 24 h. After coupling the product was washed with 4 × 10 gel volumes of the following: water, 1 mM HCl, 1 M NaHCO₃, water.

In order to remove any interfering tosyl groups in affinity experiments, portions of both the coupled gels and the non-coupled tosylated agarose were allowed to react with either (a) 0.8 M ethanolamine, pH 11, (b) 0.8 M mercaptoethanol, pH 10 or (c) 0.5 M NaHCO₃, pH 12. Preferably mercaptoethanol was used in the subsequent affinity experiments with these coupled gels. The reactions were allowed to continue for 15 h at 40 °C. Washing was done as described above. A slightly modified version of the ultraviolet method of Lindberg et al. [8] for determining agarose-bound NAD analogues was used to determine the amount of coupled adenosine phosphates. The absorbance at 290 nm was used to calculate the ligand content as absorption by tosyl groups is negligible at this wavelength. The ultraviolet spectra were taken as follows. To the sample and reference cuvettes were added 100 mg of the wet gel to be analyzed and untreated Sepharose CL-6B, respectively. To each cuvette was added 2.9 ml 87% glycerol/water solution and the cuvettes were mixed until the suspensions were homogeneous. The absorption spectra were then recorded at a scanning speed of 2 nm/s; two or three spectra were taken and the mean value was used. The amount of bound

Ahx⁶Ado5'*P* and Ahx⁶Ado(2',5')*P*₂ was found to be 5 and 3 μmol/g wet preparation, respectively (ϵ_{290} = 3300 M⁻¹ cm⁻¹). The amount of bound ligands did not change after removal of excess tosyl groups by mercaptoethanol.

Coupling of Proteins

Trypsin inhibitor and peroxidase were coupled according to the following procedure. 1 g wet tosylated agarose, washed with 0.1 M NaHCO₃ (pH 8.5 or 9.7) was added to a solution of 25 mg protein, in 0.3 ml cold 0.1 M NaHCO₃ (pH 8.5 or 9.7) and incubated on a rocking table for 24 h at 22°C. The immobilized trypsin inhibitor and peroxidase were then washed with 3 × 20 gel volumes of the following cold solutions: 0.1 M NaHCO₃, 0.5 M NaCl, 1 mM HCl, water and 0.1 M phosphate buffer.

Alcohol dehydrogenase was immobilized by the same method using 10 mg enzyme/g tosylated gel, in either 0.1 M phosphate buffer, pH 7.5, or 0.1 M NaHCO₃ buffer, pH 8.5. The bound alcohol dehydrogenase gels were washed as for trypsin inhibitor and peroxidase but without the HCl and water steps. The amount of bound trypsin inhibitor, peroxidase and alcohol dehydrogenase were determined by amino acid analysis in all cases. Cysteine was determined in the amino acid analysis by performic acid oxidation [9]. The amount of peroxidase coupled was corrected with the known amount of carbohydrate associated with the enzyme [10].

Enzyme Assays

All assays of enzyme activity were done at room temperature in 0.1 M sodium phosphate buffer (pH 7.5), by following the change in absorbance (1-cm light path). Assays of soluble lactate dehydrogenase, 6-phosphogluconate dehydrogenase and trypsin were initiated by addition of 0.05 ml enzyme solution to the following assay media (0.95 ml): for lactate dehydrogenase, 0.5 μmol pyruvate and 0.1 μmol NADH; for 6-phosphogluconate dehydrogenase, 3 μmol 6-phosphogluconic acid and 0.1 μmol NADP⁺; for trypsin, 1 μmol BzArgOEt. The absorbance changes were measured at 340 nm when assaying the dehydrogenases and at 253 nm when assaying trypsin.

The activities of immobilized enzymes were determined in a cuvette, using a magnetic stirring accessory (Zeiss) to keep the gels well suspended [11]. The total change in absorbance by the immobilized preparations were corrected by a few per cent, for the blank 'activity' of the gels as a result of stirring. Alcohol dehydrogenase was assayed by addition of 20 mg of the wet gel to 2.98 ml of 1.0 mM NAD⁺ in 0.1 M phosphate (pH 7.5). To initiate the reaction ethanol was added to a concentration of 50 mM.

Soluble alcohol dehydrogenase was analyzed in the same manner. Peroxidase was analyzed by the 4-aminoantipyrine method [12]. 5 mg wet gel was added to 2.9 ml 0.1 M phosphate buffer (pH 7.5) containing 1.25 mM 4-aminoantipyrine, 85 mM phenol and 0.85 mM hydrogen peroxide, which had been equilibrated for 3 min. The increase in absorbance was measured at 510 nm before and after addition of the gel. Soluble peroxidase was also assayed by this method.

To check the possibility of leakage of bound enzymes from the gels, the immobilized alcohol dehydrogenase preparations were analyzed in a continuous batch fashion [13]. The immobilized enzymes were suspended in a magnetically stirred batch reactor (5 ml) containing the same assay material as that described above. The reaction solution was pumped to a flow cuvette and back to the enzyme suspension. The reaction was allowed to continue until a stable increase in absorbance had been recorded. The pump was then stopped. If enzyme leakage had occurred, an increase in absorbance should have been detected. No such leakage was found. When the pump was started, an increase in absorbance was again observed.

Affinity Chromatography

The experiments were done in the cold using columns measuring 6 × 0.6 cm. The model studies were done on (a) Ahx⁶Ado5'*P*-agarose and Ahx⁶Ado(2',5')*P*₂-agarose which were treated with mercaptoethanol as described under 'Coupling of Affinity Ligands', and on (b) the same gels which were not treated. The experimental procedure followed principally that described by Brodelius et al. [14]. Mixtures of lactate dehydrogenase (2 units) and 6-phosphogluconate dehydrogenase (0.2 unit) in 0.3 ml 0.1 M phosphate buffer (pH 7.6), were applied to columns containing 0.3 g wet weight Ahx⁶Ado5'*P*-agarose and Ahx⁶Ado(2',5')*P*₂-agarose respectively, washed with the above phosphate buffer. After a 5-min equilibration, fractions (1 ml) were collected at a flow rate of 10 ml/h. Bound enzyme was eluted by addition of nicotinamide adenine dinucleotides to the above buffer.

Crude extract from beef heart was prepared as described by Ohlsson et al. [15]. 0.25 ml crude extract was added to the 0.8 M mercaptoethanol-treated Ahx⁶Ado5'*P*-agarose (0.4 g wet weight), equilibrated with 0.03 M phosphate buffer (pH 7.5) containing 1 mM cysteine. After 10 min equilibration, fractions of 1 ml were taken at a flow rate of 30 ml/h. After application of one void volume of a mixture of 0.5 mM NAD⁺ and 5 mM pyruvate in the above phosphate buffer, the flow was stopped for 4 h to allow complete ternary complex formation with the bound lactate dehydrogenase to take place. Protein

determinations, corrected for coenzyme interference, on the fractions from the affinity experiment were done according to Lowry [16]. Dodecylsulphate/polyacrylamide gel electrophoresis of lactate dehydrogenase was done according to Henriksson et al. [17] using bovine serum albumin (M_r 69000), ovalbumin (M_r 45000), soybean trypsin inhibitor (M_r 21500) and sperm whale myoglobin (M_r 17200) as marker proteins.

The capacity test on the 0.8 M mercaptoethanol-treated $\text{Ahx}^6\text{Ado5}'P$ -agarose (0.25 g wet weight) was done with repeated additions (0.25 ml) of an appropriate amount of lactate dehydrogenase in 0.1 M phosphate (pH 7.5); 5 min after each addition the gel was washed at a flow rate of 10 ml/h with 2.5 ml phosphate buffer. When leakage of activity had been observed in two subsequent washes a pulse of 1 mM NADH was applied and all enzyme that had not been washed out before was eluted. The capacity of the gel was said to be the amount that could be applied without causing any leakage of activity into the wash. The capacity of immobilized trypsin inhibitor was determined by testing the binding of trypsin to the gel as described for $\text{Ahx}^6\text{Ado5}'P$ -agarose. The bound enzyme was completely and sharply eluted with 0.2 M acetate buffer (pH 3.0).

To examine the effects of any interference by tosyl groups the affinity experiments with trypsin were done on (a) 0.3 g of tosylated Sepharose CL-6B (original tosyl content 1.15 mmol/g dry gel) which was treated with mercaptoethanol, ethanolamine or NaHCO_3 as described under 'Coupling of Affinity Ligands', and on (b) 0.3 g of untreated tosylated Sepharose CL-6B (tosyl content 1.15 mmol/g dry gel). A mixture of lactate dehydrogenase and albumin was resolved on $\text{Ahx}^6\text{Ado5}'P$ -agarose treated with mercaptoethanol as described above, as well as on untreated $\text{Ahx}^6\text{Ado5}'P$ -agarose. These experiments were started by applying 0.3 mg of each enzyme dissolved in 0.3 ml 0.1 M phosphate buffer (pH 7.5). Fractions (1 ml) were taken at a flow rate of 40 ml/h. Protein content was calculated by comparing the absorbance of the wash fractions and standard solutions of the protein studied in a double-beam spectrophotometer.

RESULTS AND DISCUSSION

Examples of Use of Ligands Coupled to Tosyl-Chloride-Activated Agarose in Biospecific Affinity Chromatography

To prove the applicability of the tosyl chloride method in biospecific affinity chromatography, we first demonstrated that the biospecificity of the immobilized affinity ligands remained unchanged. It is known from earlier work that the general ligands

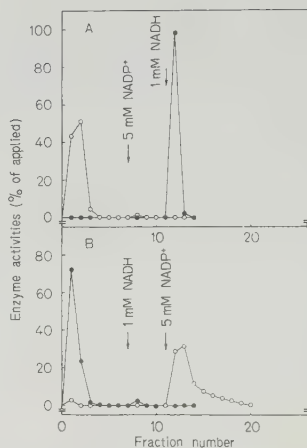


Fig. 1. Affinity chromatography of lactate dehydrogenase (●—●) and 6-phosphogluconate dehydrogenase (○—○) on (A) $\text{Ahx}^6\text{-Ado5}'P$ -agarose and (B) $\text{Ahx}^6\text{Ado(2',5')P}_2$ -agarose. The affinity gels were treated with mercaptoethanol as described under Materials and Methods. The total activities applied ($= 100\%$ in the figure) of lactate dehydrogenase and 6-phosphogluconate dehydrogenase were 2 units and 0.2 units, respectively. Applications of dinucleotides dissolved in irrigant buffer are indicated by arrows

$\text{Ahx}^6\text{Ado5}'P$ and $\text{Ahx}^6\text{Ado(2',5')P}_2$, differing only in the additional phosphate group in the latter, when used as affinity ligands, can discriminate between NAD^+ -dependent and NADP^+ -dependent enzymes [7, 14]. These two ligands were therefore thought to be suitable for model studies. The ligands were immobilized and their affinity properties tested on a mixture of lactate dehydrogenase and 6-phosphogluconate dehydrogenase. The results (Fig. 1) show, as expected, that lactate dehydrogenase bound to the gel with immobilized $\text{Ahx}^6\text{Ado5}'P$, specific for NAD^+ -dependent enzymes, but not to the gel containing $\text{Ahx}^6\text{Ado(2',5')P}_2$, which is specific for NADP^+ -dependent enzymes. All the bound lactate dehydrogenase could subsequently be specifically eluted with 1 mM NADH. In contrast, 6-phosphogluconate dehydrogenase, an NADP^+ -dependent enzyme, bound to $\text{Ahx}^6\text{Ado(2',5')P}_2$ -agarose but not to $\text{Ahx}^6\text{-Ado5}'P$ -agarose and was specifically eluted with 5 mM NADP^+ . All these results are in agreement with previous findings obtained when immobilizing the ligands via CNBr-activated agarose [14]. (The small amounts of each enzyme bound to the 'wrong ligand' can be attributed to some affinity of lactate dehydrogenase and 6-phosphogluconate dehydrogenase for $\text{Ahx}^6\text{Ado(2',5')P}_2$ and $\text{Ahx}^6\text{Ado5}'P$, respectively, in agreement with previous findings [14]).

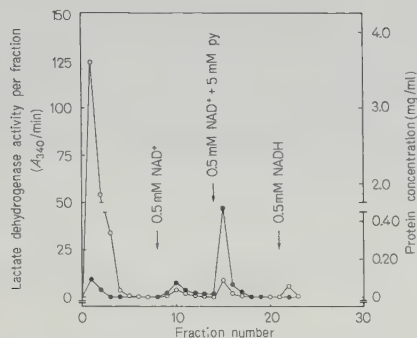


Fig. 2. Affinity chromatography on a crude beef heart extract. Total protein (O—O) and total lactate dehydrogenase activity (●—●) for each fraction is given. Applications of NAD(H) in irrigant buffer are indicated by arrows. The specific activity was calculated for fraction 15, and dodecylsulphate/polyacrylamide gel electrophoresis was run on the same fraction

The above results refer to gels which were, after binding of the ligands, treated with a nucleophilic compound, such as mercaptoethanol, to eliminate any possible interference by excess tosyl groups on the support. It had previously been observed [18] that trypsin binds noncovalently to tosyl-triethylenetetramine-Sepharose prepared by reacting tosyl chloride with a triethylenetetramine spacer bound to the matrix. To see whether any such binding took place to the tosyl groups in our tosylated agarose preparations, trypsin was applied to a gel preparation containing a large amount of tosyl groups (1.15 mmol/g dry weight). The enzyme showed weak binding to the gel as it was retarded by the gel. However, when trypsin was applied to the same gel after the latter had been treated with either alkali or nucleophilic compounds, such as mercaptoethanol or ethanolamine the enzyme did not show any binding to the gel. We thus conclude that treatment of the gels with either nucleophiles or alkali remove any tosyl groups which might cause some nonspecific weak binding. In line with this was the finding that $\text{Ahx}^6\text{Ado}5'\text{P}$ -agarose and $\text{Ahx}^6\text{Ado}(2',5')\text{P}_2$ -agarose showed sharper elution profiles after treatment with nucleophilic compound than before such treatment. As bovine serum albumin reportedly binds to toluene sulphonamides separated from the agarose matrix by a charged spacer [19] it was considered possible that it would also bind to our preparations. However, this protein was not retarded and could easily be separated from a mixture with lactate dehydrogenase on columns of $\text{Ahx}^6\text{Ado}5'\text{P}$ -agarose regardless of whether the gel had been treated with nucleophilic compounds or not.

The capacity of $\text{Ahx}^6\text{Ado}5'\text{P}$ -agarose (5 $\mu\text{molP/g}$ wet gel) was found to be 2.8 mg lactate dehydro-

Table 1. Immobilization of proteins to tosylated Sepharose CL-6B

Protein	pH for coupling	Bound protein mg/g dry support	Coupling yield %	Specific activity of bound relative to soluble enzyme
Trypsin inhibitor	9.7	74	18	—
Peroxidase	9.7	84	20	8
	8.5	58	14	11
Alcohol dehydrogenase	8.5	112	67	23
	7.5	98	59	24

genase/g wet gel. This was the amount that bound strongly. When larger amounts were applied to the gel, more could be bound but a small continuous leakage was then observed. All the enzyme bound, whether strongly or weakly, could be eluted with 1 mM NADH giving a sharp elution profile.

Lactate dehydrogenase present in a crude beef heart extract was purified on $\text{Ahx}^6\text{Ado}5'\text{P}$ -agarose (see Fig. 2). Lactate dehydrogenase was eluted using the dead-end ternary complex formation method [15]. Dodecylsulphate/polyacrylamide gel electrophoresis, run on fraction 15, gave only one band with an M_r of 36000 which corresponds well with the value of the subunit given in the literature [20]. The specific activities of lactate dehydrogenase purified by $\text{Ahx}^6\text{Ado}5'\text{P}$ immobilized via tosyl chloride or CNBr activation [15] were practically the same (90 and 85 units/mg protein, respectively, both values uncorrected for inhibition by the pyruvate-NAD complex).

Coupling of Proteins

As seen from Table 1 the tosyl chloride method can also be used for immobilization of proteins. No optimization of the conditions applied, in particular with regards to temperature, has been made at this point. As seen in the table, alcohol dehydrogenase couples in good yields even at relatively low pH. This may be because the enzyme has a high content of lysine and cysteine available for coupling [21, 22]. By comparison peroxidase carries fewer such residues [10]. For both enzymes higher relative binding yields can be expected when using lower concentration of protein in the coupling mixture.

The two alcohol dehydrogenase preparations in Table 1 showed specific activities in accordance with what has been observed for the same enzyme immobilized to CNBr-activated agarose carrying roughly the same amount of enzyme [22]. The relatively low specific activity of immobilized peroxidase is probably due to diffusional restrictions of substrate transport

as the enzyme has a high turnover rate. This is supported by the observation that immobilized peroxidase preparations with different amounts of bound enzyme had the same apparent activities.

The capacity of immobilized soybean trypsin inhibitor to bind trypsin was tested by the same method as that described for the capacity test of bound $\text{Ahx}^6\text{Ado}^5\text{P}$ for lactate dehydrogenase. It was found that 60 mg of trypsin/g dry support was bound. The enzyme could subsequently be easily eluted with 0.2 M acetate (pH 3.0). Only active trypsin bound to the inhibitor-carrying gel. Inactive forms showed no retardation at all on the columns. The results are in agreement with previous affinity chromatography studies with immobilized soybean trypsin inhibitor [4].

Conclusion

From the affinity chromatography studies described, as well as those on immobilized proteins, the following conclusions can be drawn. The tosyl chloride method when applied in affinity chromatography appears useful as the same affinity behaviour is found, if for example $\text{Ahx}^6\text{Ado}^5\text{P}$ -agarose or $\text{Ahx}^6\text{Ado}^5(2',5')\text{P}_2$ -agarose via CNBr or tosyl chloride activation are used. Likewise, proteins and enzymes can be coupled covalently showing good retained activities. Further, in some cases there seem to be advantages of the tosyl chloride method over the CNBr method, in particular when the affinity ligand to be coupled is stable. For example, the N-C and S-C bonds assumedly formed between ligand and matrix on immobilization of amino or thiol compounds, are very stable. In addition, no extra charged groups are introduced on immobilization, and on coupling of thiol compounds the linkages are unchanged. On immobilization to CNBr-activated supports, on the other hand, the linkages formed are somewhat labile [23] and additional charged groups are introduced [24] which are known to cause non-specific adsorption in certain affinity systems [25]. Further, to the authors knowledge, stable linkages between thiol-group-containing compound and CNBr-activated gels have not been described. Compared to the oxirane method [24], tosylation of gels is rapid, can easily be followed by ultraviolet measurements and cross-linking of the gel probably does not occur. Besides cross-linked or non cross-linked agarose the method is also applicable to other hydroxyl-group-

containing supports; furthermore it appears likely that even more reactive sulfonyl chloride derivatives can be used in analogous fashion (see Note added in proof).

We would like to thank the Swedish Natural Science Research Council and the National Swedish Board for Technical Development for their generous support. We thank Drs T. A. D. G. H. Griffin and S. Birnbaum for valuable discussions.

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Note Added in Proof. More details on the various parameters affecting immobilization yields can be found in *Acta Chemica Scandinavica* [K. Nilsson, O. Norrlov, and K. Mosbach, in the press]. In addition, more recently we found that tosylation of the gels can be carried out efficiently in dry acetone instead of dioxane.

Also a number of other hydroxyl group-containing supports were successfully used and applying other sulfonyl chloride derivatives very high coupling of added enzymes after incubation for a few hours at pH 8 and 4°C was obtained with specific activities of up to 60%.

p-Toluenesulfonyl Chloride as an Activating Agent of Agarose for the Preparation of Immobilized Affinity Ligands and Proteins. Optimization of Conditions for Activation and Coupling

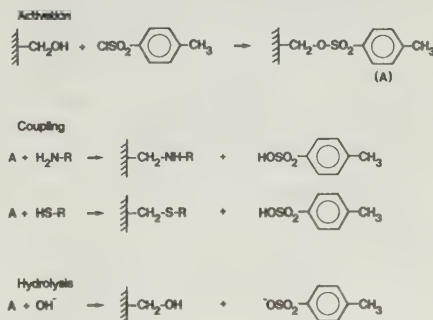
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A new immobilization technique suitable for the binding of biomolecules to polysaccharide supports, such as agarose beads, is described. It involves treatment of agarose with *p*-toluenesulfonyl chloride (tosyl chloride) with the formation of *p*-toluenesulfonic ester (tosylation). This reaction is rapid and can be controlled to give a range of substitutions up to very high levels. The tosylated agarose obtained shows excellent long-term stability and the swelling properties of the gel are similar to those of untreated agarose indicating that no cross-linking of the support occurs during tosylation. The degree of substitution of agarose is easily monitored by UV measurement of the appearance of *p*-toluenesulfonate. Addition of nucleophiles, such as amino or mercapto group-containing compounds, leads to efficient displacement of the tosyl groups. With β -alanine and β -mercaptopropionic acid as model compounds the degree of coupling was investigated as a function of time, temperature and pH. High degrees of substitution (in the range 6–10 $\mu\text{mol/g}$ wet gel) were obtained within 10 h at pH 8–10 and room temperature. Higher yields were obtained on prolonged reaction time, more alkaline conditions, and higher temperature. It was concluded from titration data that ligands were coupled without the introduction of any additional charged groupings and that on coupling of thiol compounds uncharged linkages were formed between ligand and support. Coupling in organic solvent of ligands not soluble in water gave high yields of coupled product. Some results of the coupling of biomolecules, such as affinity ligands and enzymes, to agarose are given.

Probably the most common procedure for binding to polysaccharide supports involves activation by cyanogen bromide,³ while another frequently used polysaccharide support carries epoxy groups.⁴ Although these procedures have proved successful, they may have drawbacks under certain conditions (see Results and Discussion).

We, therefore, feel that the more coupling methods that are available the better the chances of finding an immobilization procedure tailor-made for a particular purpose. We have found tosylated polysaccharides to be suitable alternative supports for immobilization. It is known from the chemistry of soluble saccharides that reaction of hydroxyl groups with *p*-toluenesulfonyl chloride (tosyl chloride) forms the corresponding esters (tosylates) in which the tosyl groups have excellent leaving properties in reactions with nucleophiles giving stable linkages between ligand and saccharide carbon.^{5,6} This finding



Scheme 1.

A number of immobilization techniques leading to covalent attachment of biomolecules, such as affinity ligands¹ or enzymes,² are known and applied.

prompted us to try to develop a procedure suitable for the binding of biomolecules to polysaccharides. The pathway of these reactions are analogous to those of tosylates of soluble saccharides^{5,6} and are thought to involve the steps given in Scheme 1.

In this study the chemistry involved is discussed together with investigations of various variables playing a role in ligand binding.

EXPERIMENTAL

N,N-Dimethylformamide (DMF, spectroscopic grade), diglyme (diethyleneglycol dimethyl ether) *p.a.*, acetone *p.a.*, glycerol (87%) *p.a.*, dried dioxane (max. 0.01% H₂O), hexylamine *p.s.* and *p*-toluenesulfonic acid *p.a.* were from Merck, Darmstadt, West Germany. Sepharose 6B and Sepharose CL-6B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Histamine hydrochloride (98%) and *p*-toluenesulfonyl chloride (98%) were from Aldrich-Europe, Beersee, Belgium. Pyridine was bought from BDH Chemicals Ltd., Poole, England. β -Alanine, γ -aminobutyric acid (99%) and 1,6-hexanediamine were from Sigma Chemicals, St. Louis, Mo., U.S.A.

ϵ -Aminocaproic acid (99%) was from Kebo AB, Stockholm, Sweden, β -mercaptopropionic acid (99%) from Fluka AG, Buchs, Switzerland, Omnifluor from New England Nuclear, Albany St., Boston, Mass., U.S.A. and Triton-X-100 (scintillation grade) from Eastman Kodak Company, Rochester, N.Y., U.S.A. (Ring-2-¹⁴C)-histamine dihydrochloride (2.2 GBq/mmol in 0.25 ml) was from the Radiochemical Centre, Amersham, U.K. All other chemicals were of analytical grade and used as supplied. Redistilled water was used throughout.

Agarose gel beads. Gels were washed on a glass filter funnel and equilibrated in each washing medium for some minutes before the suspensions were gently sucked dry by vacuum until a wet gel cake was obtained (care was taken to avoid drawing air into beads). In order to determine the degree of swelling of the gel beads they were weighed, freeze-dried for about 24 h and then reweighed.

Activation (tosylation). The following procedure holds for all activations and can be scaled up or down if needed. When the reaction was done in diglyme or acetone we used the same procedure as that described below. Unless otherwise stated, the dioxane used in the washings was of analytical grade.

Wet Sepharose CL-6B was washed with 2 $\times$ 10 gel volumes of water, water-dioxane 3:1 (v/v), water-dioxane 1:3, dioxane and finally with 3 $\times$ 5 gel volumes of dried dioxane (containing less than 0.01% water). The purpose of this washing procedure was to get rid of water in accordance with similar washing

procedures⁷ (water may hydrolyze the tosylating agent, tosyl chloride).

The above Sepharose (7 g wet weight) was then transferred to a round-bottomed flask containing 1 g of tosyl chloride dissolved in 2 ml of dried dioxane. With magnetic stirring 1 ml of pyridine was added dropwise for about 1 min. After 1 h at room temperature, the gel was washed with 2 $\times$ 10 gel volumes of dioxane and then gradually transferred back to water by reversing the washing scheme described above. The gels were stored in distilled water at 4 °C until used.

In all tosylations the same 1:1 ratio (w/v) of tosyl chloride to pyridine as that used above was applied. Preparations A and B were made by taking aliquots after 10 and 30 min, respectively, from the reaction mixture and washing them immediately with dioxane, as described above. Preparation G was made in exactly the same way as preparation C, using Sepharose 6B.

Coupling procedures. The following procedure was used for coupling amino or mercapto group-containing ligands: The ligand was dissolved in 0.5 ml of 0.5 M sodium bicarbonate buffer of pH appropriate for coupling (see Results and Discussion section for further details) and kept at 4 °C. A wet tosylated agarose preparation was washed with the above cold bicarbonate buffer, gently sucked dry, after which 1.75 g was added to the solution with dissolved ligand. The pH was checked and corrected, if necessary, by addition of 3 M NaOH or 3 M HCl. The mixture was placed in a water-bath with a shaker for coupling at 40 °C. A rocking table was used when coupling was carried out at 4 or 20 °C. After coupling the support was washed with 4 $\times$ 10 gel volumes of the following: Distilled water, 1 mM HCl, 1 M Na₂CO₃, distilled water.

Hexylamine was coupled in DMF by dissolving 0.16 ml of hexylamine in 0.34 ml of DMF and adding 1.75 g wet tosylated agarose (preparation C), which had first been carefully washed free from water with DMF. Coupling was done in a water-bath with a shaker at 40 or 60 °C. The products were carefully washed with DMF and twice with acetone in order to wash away unbound ligand.

UV-measurements and determination of tosyl group content. Spectra were recorded between 320 and 200 nm with a Beckman double-beam spectrophotometer in cuvettes with 1 cm light path. A glycerol-water solution (87:13 w/v) was used to get stable and homogeneous gel suspensions. To the sample and reference cuvettes were added 100 mg of the wet gel to be analyzed and untreated Sepharose CL-6B gel, respectively.

To each cuvette was added 2.9 ml of 87% glycerol-water solution. The cuvettes were mixed for 3-4 min or until the suspensions were homogeneous. The absorption spectra were then recorded at a scanning

speed of 2 nm s^{-1} . 2–3 spectra were taken and the mean value was used. The extinction coefficient for ethyl tosylate at 261 nm, $480 \text{ M}^{-1} \text{ cm}^{-1}$,⁸ was used to calculate the tosyl group content in wet tosyl gels.

When analyzing the hydrolysate from 1 M NaOH treated tosylated gels, the UV spectra were taken with 1 M NaOH as reference. To calculate the amount of *p*-toluenesulfonic acid in the hydrolysate, the extinction coefficient at 260 nm for the acid in 1 M NaOH was determined from absorption spectra on standard solutions of the acid. The absorption spectra of *p*-toluenesulfonic acid was practically the same in 87% glycerol, distilled water and 1 M NaOH.

The sulfur, nitrogen and chloride contents of freeze-dried tosylated gels were determined by elemental analysis. Gels washed as described under "Activation" and gels which had also been washed carefully with chloroform and acetone were analyzed. The tosyl group content was calculated from the sulfur content and the amount of tosyl groups per disaccharide subunit (mol. wt. 306) was then calculated.

Determination of the amount of coupled ligands. The amount of immobilized ligands was determined by titration. In addition, elemental analysis (on hexylamine preparations and on some of the β -alanine preparations) and radioactivity measurements (of histamine preparations only) were also done. The degree of swelling was determined as described above and it was found that the swelling of the tosylated preparations was not significantly changed after coupling of ligands.

Titration was done with 0.2 M NaOH at a low speed ($0.25 \mu\text{s}^{-1}$) in a titration assembly with automatic recording of the titration curve. The gels to be analyzed were washed in 1 M KCl on a glass filter funnel, as described under "Agarose beads". To 1 g of the wet gel, placed in a titration vessel, appropriate volumes of 1 M KCl and 1 M HCl were added to get the desired titration volume and starting pH, respectively.

Titration were carried out at a pH ranging from 2.0 to 11.5.

Immobilized histamine was determined by radioactivity measurements of the hydrolysate of the freeze-dried preparations, as described by Nilsson and Mosbach.⁹

RESULTS AND DISCUSSION

Activation (tosylation) of agarose. The activation was achieved mainly with dioxane as solvent, but later we found diglyme (diethyleneglycol dimethyl ether) and acetone to be good alternatives. In order to get high yields of tosyl groups on the polymer, conditions as water-free as possible had to be used during tosylation, since water rapidly hydrolyzes the tosyl chloride. We therefore used dried dioxane (containing less than 0.01% water) as solvent to get constant conditions and reproducible results.

Table 1 gives the variation of the amount of tosyl groups incorporated per g of dry gel with the amount of tosyl chloride and reaction times used in tosylation. The amount of tosyl groups was determined by elemental analysis of the sulfur content of the different freeze-dried gels. It is clear from Table 1 that on application of 2.5 g tosyl chloride g^{-1} dry agarose (=17.5 ml wet), the reaction is fairly rapid. After only 10 min as much as 0.62 mmol tosyl groups were introduced per g dry product. Extending the contact time to 60 min gave a product with 1.15 mmol tosyl groups g^{-1} . This is a high degree of substitution, higher than the oxirane content obtained after 8 h reaction of Sepharose 6B with 1,4-butanediol diglycidyl ether under optimal conditions (about 1 mmol g^{-1} dry gel),⁴ and it is

Table 1. Tosylation of agarose. For all preparations Sepharose CL-6B was used except for sample G where Sepharose 6B was used. Calculations were done as described under Experimental.

Preparation	Tosylation time min	Amount of tosyl chloride used in tosylation g g^{-1} of dry agarose	Found S % (w/w)	Amount of tosyl groups in product mmol g^{-1} of dry weight	Number of tosyl groups per disaccharide unit
A	10	2.5	2.01	0.62	0.21
B	30	2.5	3.01	0.94	0.34
C	60	2.5	3.70	1.15	0.43
D	60	5.0	4.83	1.51	0.60
E	60	1.25	1.98	0.62	0.21
F	60	0.5	0.60	0.19	0.06
G	60	2.5	3.29	1.03	0.38

much higher than the substitution of commercially available CNBr- or epoxy-activated preparations (0.4 mmol cyanate ester groups g^{-1} dry gel¹⁰ and 0.3–0.4 mmol epoxy groups g^{-1} dry gel,¹¹ respectively).

A fairly linear increase of the amount of tosyl groups in the product was obtained on increasing tosyl chloride concentrations to 2.5 g g^{-1} dry gel.

The tosylation reaction is known to have marked selectivity for primary hydroxyls in sugars.^{5,12} The repeating disaccharide subunit in agarose, consisting of 3-linked D -galactopyranose and 4-linked 3,6-anhydro- L -galactopyranose, with a molecular weight of 306, contains one primary hydroxyl when the polymer is not cross-linked.^{13,14} In preparation D with 1.5 mmol of tosyl groups g^{-1} gel, this would mean that over 60% of the original primary hydroxyls were tosylated (compare with Table 1). Since we have normally used cross-linked agarose the percentage of substituted hydroxyl groups was even higher.

Owing to the high stability of the cross-linked agarose used, the degree of swelling of all the gels remained almost constant and was roughly equivalent to that of the original agarose preparation. The only exception was the highly substituted preparation D, which swelled about 10% less than the lower substituted gels calculating from the agarose content in the products. This was probably due to its high level of hydrophobic groups, which are known to cause shrinkage of agarose gels in water.¹⁵ (Almost 25% of the dry weight was made up of tosyl groups).

To get optimal coupling of amines it was not necessary to use more than 2.5 g g^{-1} tosyl chloride g^{-1} dry gel in the tosylation giving 1.15 mmol of tosyl groups g^{-1} dry gel (Fig. 1). This amount of reactive groups is in agreement with the epoxy method where about 1 mmol epoxy groups g^{-1} is required for optimal yields.⁴

β -Mercaptopropionic acid was, as expected, more reactive than β -alanine. The former required gels of lower activation level and it was not necessary to activate with more than 1.25 g g^{-1} tosyl chloride g^{-1} dry gel to get good binding.

To ascertain whether non-cross-linked agarose can be tosylated by the method described above, Sepharose 6B was allowed to react with tosyl chloride. As seen in Table 1 (preparation G) the degree of tosylation was high. No optimization has been carried out. In one experiment, β -mercaptopropionic acid was coupled to the

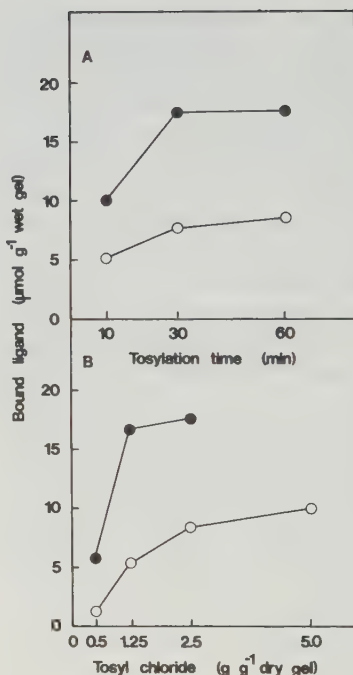


Fig. 1. Coupling of β -alanine (○) and β -mercaptopropionic acid (●) to different tosyl Sepharose CL-6B preparations as made when using (A) different times for tosylation and (B) different amounts of tosyl chloride during tosylation. In (A) 2.5 g g^{-1} tosyl chloride g^{-1} dry Sepharose was used and the tosylation time was 60 min in (B). Coupling was carried out for 20 h at 40 °C as described under Experimental with 0.4 M ligand at pH 10.6 (β -alanine) and pH 10.0 (β -mercaptopropionic acid). The amount of bound acid was determined by titration.

activated support and the amount bound was 12 $\mu\text{mol g}^{-1}$ wet preparation (coupling done at pH 10, 40 °C for 40 h with 0.4 M ligand) and should be compared with 18 μmol with Sepharose CL-6B under similar conditions. Other commercially available agarose preparations, such as Sepharose 4B, were not studied in this investigation. They too can probably be activated by the method described here.

UV measurements on tosyl gels. The UV spectra of tosyl chloride, -acid and -esters show characteristic

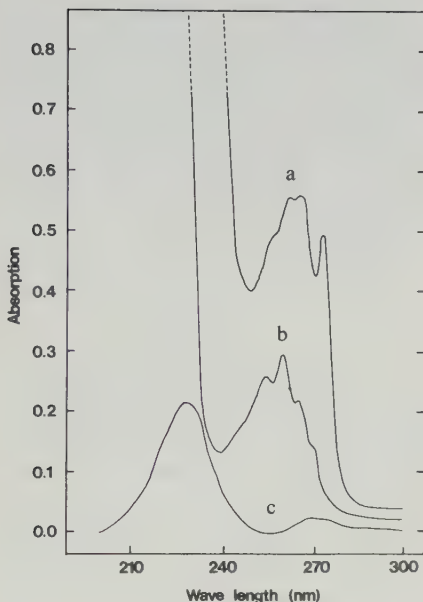


Fig. 2. UV spectra of (a) preparation A from Table 1, (b) the hydrolysate from 1 g wet gel (= 50 mg dry) of sample A and (c) preparation A after treatment with 1 M NaOH for 60 h at 60 °C. The total volume of the hydrolysate was 50 ml and spectra were taken directly on the hydrolysate in a double-beam spectrophotometer using 1 M NaOH as reference. Spectra on the gel samples were taken as described under Experimental.

absorption maxima.⁸ UV measurements were therefore used to confirm that the sulphur content determined by elemental analysis was that of the tosyl ester. Ethyl tosylate taken as an example of the latter has a spectrum differing from the chloride and acid and has absorption maxima at 232, 261 and 273 nm, minimum at 249 nm and has a shoulder at 267 nm. The same maxima and minimum were also found for the spectra of the tosylated gels indicating formation of tosyl esters. Also, the relative intensities of the maxima at 273 and 261 nm and of the minimum at 249 nm were similar in the tosylated gel and in the ethyl tosylate spectra.

The spectrum of a tosyl gel, preparation A of Table 1, is given in Fig. 2 (curve a). This figure also gives the spectrum of the filtrate (curve b) obtained on hydrolysis of preparation A, as well as the

spectrum of the gel remaining after hydrolysis (curve c).

The spectrum of the hydrolysate from the gel corresponds to that of *p*-toluenesulfonic acid. The amount of acid in the hydrolysate was calculated and found to correspond with the tosyl content given in Table 1 based on elemental analysis of sulfur.

As can be seen in Fig. 2, nearly all the original tosylate groups on the gel were hydrolyzed. Elemental analysis of the gel indicated that about 0.1% (w/w) sulfur remained on the support. Absorption measurements at 261 nm can be used for the determination of the tosyl content of the gels. Fair correlation ($\pm 5\%$) with the elemental analysis were found except for the very highly substituted gels (preparation C and D) where flattening of the spectra occurs resulting in about 15 and 30% lower values than obtained with elemental analysis. (Such flattening of the absorption spectrum of strongly absorbing particles has been described by Duysens.)¹⁶

To check further that the sulfur was not to any great extent due to adsorbed *p*-toluenesulfonic acid, the tosyl gels were carefully washed with acetone and chloroform and analyzed for sulfur again: The decrease in sulfur was less than 5%. Because of this, and the above UV measurements, it was concluded that the sulfur content determined by elemental analysis corresponded almost entirely to tosyl ester.

The gels were analyzed for nitrogen and chloride to ascertain whether any of the side reactions most frequently reported for soluble saccharides, such as displacement of tosyl by pyridine or chloride, had occurred during activation.⁵ At most, traces were found, indicating that these reactions do not take place to any significant extent. Thus, unlike the CNBr and epoxy methods (giving unreactive carbamates² and cross-linking,⁴ respectively), the activation procedure described here does not seem to lead to unwanted side-effects.

Stability of tosyl gels. The tosyl preparations were very stable when stored in the cold in the wet state in distilled water and could be used as required. Preparation C, for example, showed a 25% decrease in the coupling yield of β -mercapto propionic acid (coupling done at 40 °C for 15 h at pH 10 with 0.4 M ligand) after storage in the cold for 3 months in distilled water. It is probable that storage under slightly acidic conditions (pH 4–5) or freeze-drying would render the preparations even more stable.

The stability of the tosyl esters decreases with

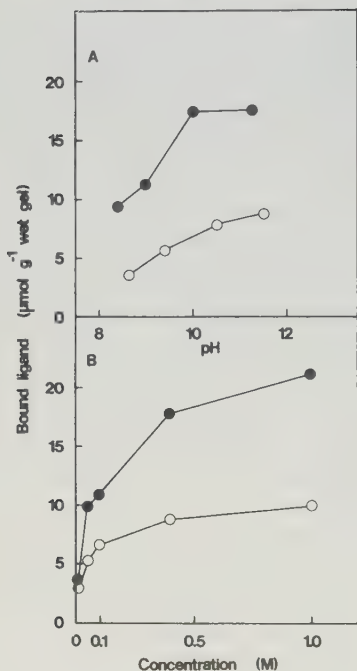


Fig. 3. Dependence on pH (A) and amount of ligand (B) during coupling of β -alanine (○) and β -mercaptopropionic acid (●) to tosyl Sepharose CL-6B (preparation C). Couplings were done for 20 h at 40 °C as described in Experimental. In the pH dependence experiment 0.4 M ligand was used and in the concentration dependence experiment the pH was 10.6 (β -alanine) and 10.0 (β -mercaptopropionic acid). In a coupling volume of 2.5 ml 1.75 g wet gel was used. The amount of bound acid was determined by titration.

increasing pH, and when the highly substituted preparation C was treated at 40 °C for 15 h at pH 10 and 13, 12 and 40% of the tosyl groups were hydrolyzed, respectively. As already mentioned, treatment with 1 M NaOH hydrolyzes nearly all the tosylates on the gel (Fig. 2).

Coupling of small ligands to tosylated agarose. β -Alanine and β -mercaptopropionic acid were used as model substances to determine how the coupling of amines and thiols depends on pH, temperature, reaction time and reagent concentration. Preparation C in Table 1 was used as tosylated gel.

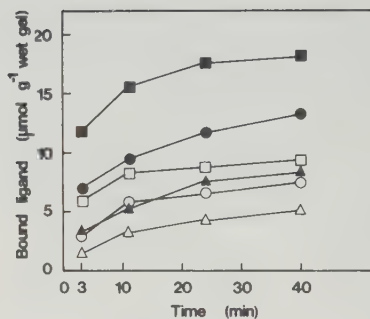


Fig. 4. Dependence of coupling time and temperature for the coupling of β -alanine (open symbols) and β -mercaptopropionic acid (closed symbols) to tosyl Sepharose CL-6B (preparation C). Three different temperatures were studied: 4 °C (△), 22 °C (○) and 40 °C (□). The ligand concentration was 0.4 M and pH 10.6 and 10.0 were used for β -alanine and β -mercaptopropionic acid, respectively. In a coupling volume of 2.5 ml 1.75 g wet gel was used. Coupling procedures are given under Experimental. The amount of bound ligand was determined by titration.

As can be seen (Figs. 3 and 4) the thiol-containing compound gave higher coupling yields throughout the experiments. The reactivity of tosylated agarose towards these nucleophiles seems to be of the same order as that reported for epoxy-activated agarose.¹⁷ As for most coupling methods, the yield is dependent upon pH (Fig. 3 A). As expected from the pK_a values of the thiol and amine (9.5 and 10.3), it is not necessary to use higher coupling pH than 10 for the thiol, while a higher pH may be used with advantage for the amine. This is not the case for an amine with a lower pK_a , such as histamine, for which pH 10 is high enough to get a maximum yield.

Fig. 3 B shows the dependence of the degree of coupling on the concentration of the ligand. The curves obtained for the amine and thiol have the same shape and show a continuous levelling off with increasing concentration of ligand in the coupling solution. Relatively high yields of coupled ligand were obtained with low ligand concentrations in the coupling mixture.

As seen from Fig. 4, the coupling yield was higher when coupling was done at higher temperatures. A fair degree of coupling occurred within 3 h at 40 °C. The lower rate of coupling at lower temperatures could be partly compensated for by extension of the

Table 2. Coupling of 0.5 M hexylamine in dimethylformamide to tosylated agarose (preparation C in Table 1). The coupling time was 40 h. 1.75 g gel was used in a coupling volume of 2.5 ml. Coupling procedures and determination of tosyl and hexylamine are described in Experimental.

Temp. for coupling °C	Coupled hexyl-amine ^a	Remaining tosyl groups ^a	Original amount of tosyl groups ^a
40	0.32	0.80	1.15
60	0.76	0.38	1.15

^ammol g⁻¹ dry weight preparation.

coupling time. The coupling efficiency was about the same for the amine at 20 °C as for the thiol at 4 °C.

In the highest substituted thiol preparations about 30% of the tosyl groups were replaced by ligand (Fig. 3). The degree of displacement was much higher (Fig. 1) for the gels with lower tosyl content (about 60%). As discussed in more detail elsewhere, it was not found necessary in our affinity studies¹⁸ to remove all remaining tosyl groups from the gels. However, after coupling of the affinity ligand, the support can be allowed to react, if necessary with mercaptoethanol, which does not introduce any additional charges, or with ethanolamine to replace interfering tosyl groups.

Another amino compound, histamine, was also coupled to preparation C and the coupling yield was studied with respect to the same parameters as for β -alanine. The coupling behaviour was found to be roughly the same as that for β -alanine. The coupling yield with histamine was about 10–20% higher than that with β -alanine, possibly because some additional coupling took place through imidazole nitrogen. Titration curves obtained with the histamine gels support this view.

Hexamethylenediamine, γ -aminobutyric acid and ϵ -aminocaproic acid were also coupled to preparation C (Table 2). The coupling yields were practically the same as those for β -alanine. Immobilized hexamethylenediamine could, in principle, act as a spacer and could be used for binding other amino compounds after treatment with glutaraldehyde.¹⁹

Titration of coupled products. The coupling scheme outlined in the introduction is a nucleophilic displacement by the ligand of the tosyl group on the support. Thus, a primary amino group yields a

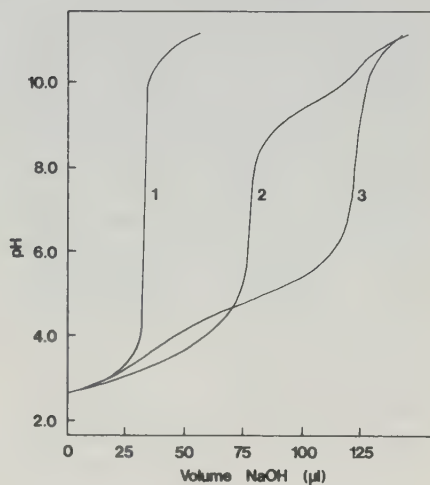


Fig. 5. Titration of (1) tosyl agarose (preparation C), (2) β -alanine agarose and (3) β -mercaptopropionic acid agarose with 0.2 M NaOH. The β -alanine and β -mercaptopropionic acid-containing gels were obtained from preparation C by coupling 0.4 M of each ligand for 24 h at 40 °C at pH 10.5 by the procedure described in Experimental. The amount of wet gel used in the titration experiment was 1.0 g.

secondary amine, a thiol group a thioether and a carboxyl group an ester when coupled to tosylated agarose. Carboxyl groups are not likely to couple to any significant extent compared with the much more reactive amino and thiol groups. In the case of β -alanine, two charged groups in equal amounts are expected to be found on the gel; in contrast the thiol compound β -mercaptopropionic acid is expected to give only one charged group on the gel. This is in accord with the titration curves obtained for these two compounds (Fig. 5). The curve for immobilized β -alanine (curve 2) indicates equal amounts of two charged groups, one with pK_a around 3.8 (carboxyl, 9 $\mu\text{mol g}^{-1}$ wet gel) and one with pK_a around 9.5 (amine, 9 $\mu\text{mol g}^{-1}$ wet gel). Curve 3, obtained with immobilized β -mercaptopropionic acid, indicates only one charged group with a pK_a of about 4.7 (carboxyl, 18 $\mu\text{mol g}^{-1}$ wet gel).

No titrable groups could be detected in tosylated gels (curve 1) indicating that no charges were introduced on tosylation. The titration data thus indicates that a ligand can be attached by this

method without the introduction of any additional charged groups. This contrasts, for example, with the CNBr-method, which besides isoureas results in additional charged, non-characterized nitrogen-containing moieties on coupling of ligands.^{10,20,21} Further, it was possible to get uncharged linkages between ligand and matrix on coupling of a thiol compound to tosylated gels. The N—C and S—C bonds most likely formed upon immobilization to tosylated agarose are expected to be extremely stable (the S—C bond is very resistant under acidic or alkaline conditions²²). In addition, nucleophiles are not likely to cause leakage of ligands, which is in contrast with CNBr formed bonds, which also are chemically unstable.²³

Coupling in organic solvent. Many ligands of interest in affinity chromatography are not readily soluble in water and to get high degrees of coupling of these ligands it is, therefore, advantageous if coupling can be carried out in organic solvents. To show the possibility of coupling under non-aqueous conditions, hexylamine was coupled to preparation C at two different temperatures in DMF. As is seen from Table 2 a very high degree of substitution was obtained at 60 °C with about 70 % of the tosyl groups being replaced by hexylamine. It is clear from the table that the decrease in tosyl content is almost completely "matched" by bound hexylamine. This is in agreement with the proposed coupling pathway (nucleophilic displacement) and indicates that no side reactions, such as hydrolysis or S—O bond fission, take place.

Coupling of affinity ligands and enzymes using preparation C. A number of biomolecules were coupled covalently by nucleophilic displacement to agarose preparations substituted with tosyl groups. In one series of experiments analogues of 5'-AMP and 2'-ADP were bound by their terminal amino groups to the polysaccharide support. It could be shown that from a mixture of lactate and 6-phosphogluconate dehydrogenase the immobilized AMP analogue showed bio-affinity only for NAD⁺-dependent lactate dehydrogenase, whereas the immobilized ADP analogue showed affinity only for the NADP⁺-dependent 6-phosphogluconate dehydrogenase. Furthermore, the immobilized AMP analogue (5 $\mu\text{mol g}^{-1}$ wet gel) was applied for the single step purification of lactate dehydrogenase from crude beef heart extract.

To demonstrate the immobilization of proteins, soybean trypsin inhibitor was immobilized to tosylated agarose (75 mg/g dry product), tested as

affinity chromatography material and shown to bind 60 mg of trypsin per g dry gel. Horse radish peroxidase and horse liver alcohol dehydrogenase were used as model enzymes. Although no optimization had been attempted, the former (approximately 70 mg/g dry support) had a coupling yield of approximately 18 % with a specific activity (relative to soluble enzyme) of approximately 10 %, whereas approximately 60 % of alcohol dehydrogenase was coupled (approximately 100 mg/g dry support) with a specific activity of approximately 25 %.

CONCLUSION

p-Toluenesulfonyl chloride has been shown to be suitable for the preparation of agarose bound ligands. Activation of the gel, leading to substitution with tosyl groups, is rapid, gives predictable levels of activation and the activated gel obtained is stable in distilled water. The swelling properties of the activated gels are practically not affected. By measuring the UV-absorbance one can easily determine the degree of substitution of the gel which is much more time-consuming, if at all possible, with most other activation methods. Water-insoluble ligands can be coupled in organic solvents. Most important, stable, uncharged, "direct" linkages between ligand and matrix are obtained without the introduction of any additional groups, thereby making it possible to devise highly specific affinity chromatographic systems. The coupling efficiency with amino or mercapto group-containing compounds compares well with other methods known.

Acknowledgement. The financial support by the Swedish Natural Science Research Council is gratefully acknowledged.

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Received August 7, 1980.



IMMOBILIZATION OF ENZYMES AND AFFINITY LIGANDS TO VARIOUS HYDROXYL GROUP
CARRYING SUPPORTS USING HIGHLY REACTIVE SULFONYL CHLORIDES

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Received July 28, 1981

SUMMARY

A facile method for the activation of hydroxyl group carrying supports such as agarose, cellulose, diol-silica, glycophasse-glass or hydroxyethyl methacrylate gels with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) is described. On reaction of the resulting tresylate containing supports with different enzymes or affinity ligands at neutral pH, overnight and at 4 °C, coupling yields and retained specific activities of up to 80 and 50 %, respectively, were obtained. The bound ligands showed excellent affinity properties. Thus, N⁶-(6-aminohexyl)-AMP coupled to diol-silica completely separated a mixture of albumin, lactate dehydrogenase and alcohol dehydrogenase in less than 15 min when the technique of high performance liquid affinity chromatography was employed.

INTRODUCTION

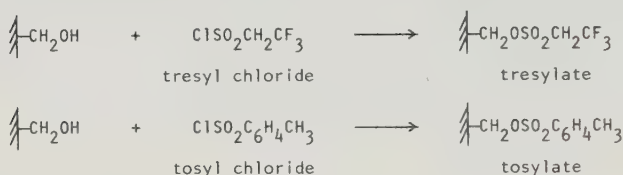
Recently a new method of activating hydroxyl group carrying supports using p-toluenesulfonyl chloride allowing subsequent immobilization of proteins and affinity ligands was described (1-3). This method was shown to have advantages over presently used ones since ligands are bound directly to the carbon atoms of the support and no side reactions occur during activation and coupling. In the literature a number of derivatives of sulfonic esters have been reported to be more reactive than these tosylates including p-nitrobenzenesulfonate, trifluoromethanesulfonate and 2,2,2-trifluoroethanesulfonate (tresylate) (4). In this report we wish to describe an immobilization method based on the latter compound as it allows efficient coupling under very mild conditions and to a number of different supports including those used for high performance liquid chromatography. The reactions involved in immobilization of biomolecules using

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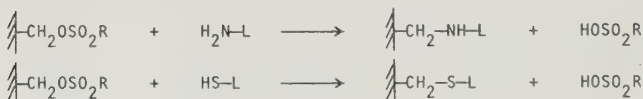
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tresyl chloride or tosyl chloride are believed to be as given in the scheme below:

Activation:



Coupling:



(R = CH₂CF₃ or C₆H₄CH₃; L = ligand or enzyme)

MATERIALS AND METHODS

Activation with tresyl chloride was performed at room temperature, principally as described for activation with tosyl chloride (1,2). Sepharose (4B, CL-4B or CL-6B, Pharmacia) was transferred to dry acetone via the following washing steps on a glassfilter funnel: 10 gel volumes of dist. water, 30:70, 60:40 and 80:20 of acetone p.a.:water (V/V), 2x10 volumes of acetone and finally 3x5 volumes dried acetone (dried over molecular sieve, 100 g/3 l acetone). The gel (32 g) was then transferred to a beaker containing 0.1 gel volume dry acetone and pyridine (2 ml/ml tresyl chloride). Under vigorous magnetic stirring 0.2-1.0 ml tresyl chloride (>97 %, Fluka) was added dropwise for 1 min to the gel-suspension. The reaction was continued for 10 min. The gel was then washed with 2x10 gel volumes acetone, 10 volumes of 30:70, 50:50, 70:30 and 85:15 1 mM HCl:acetone and finally with 2x10 volumes of 1 mM HCl and stored at 4 °C until used. The entire activation procedure can be completed within 1.5 h.

Cellulose (Sigmacell, type 100) was activated by the same procedure after swelling in 1 M NaOH for 1 h. To facilitate stirring during activation 0.5 gel volumes of acetone was added.

Diol-silica (1,2-dihydroxy-3-propoxypropylsilyl-silica), prepared from LiChrospher Si 500 (Merck) as described previously (5), LiChrosorb Diol (5 µm, Merck) and glycopase-glass (Glycophase-GTM/CPG-40, 5-10 µm, Corning) were initially washed with 90:10 acetone:water (V/V), then washed with dried acetone and treated with tresyl chloride as above, except for using 12 ml of dry acetone/g dry gel to facilitate stirring. After washing of the gels as described above they were ready for use. Alternatively, they could be washed with acetone and sucked dry for storage.

Hydroxyethyl methacrylate (Spheron^R P 100,000, Koch-Light) was treated as the silica gels, however 1 gel volume acetone was added to the settled gel to allow proper stirring during activation.

Coupling. Trypsin (bovine pancreas, type III, Sigma), trypsin inhibitor (soybean, type 1-S, Sigma), albumin (bovine, fraction V, Sigma) and N⁶-(6-aminoethyl)-AMP

(Sigma) were dissolved in cold coupling buffer (either 0.2 M sodium bicarbonate or 0.2 M sodium phosphate, + 0.5 M sodium chloride). One volume of settled activated gel was briefly washed with the cold coupling buffer and added to one volume of ligand solution. Coupling proceeded at 4 °C with gentle agitation. Coupling was terminated by washing with 3x10 gel volumes of coupling buffer, 0.2 M sodium acetate, pH 3, containing 0.5 M sodium chloride, 0.5 M sodium chloride, dist. water and coupling buffer. Hexokinase (yeast, type III, Sigma) was coupled in 0.2 M Hepes buffer, pH 7.0 containing 15 mM MgCl₂, and washed as above omitting the acetate buffer. Concanavalin A solution (14.5 mg in 0.5 ml saturated sodium chloride, Miles), was added at 4 °C to 1.1 g wet tressyl Sepharose 4B, which had been rapidly washed with buffer (0.2 M sodium phosphate + 0.5 M sodium chloride, pH 7.5). The gel was washed as recommended for CNBr coupled Concanavalin A (6).

Protein content was determined by amino acid analysis and bound AMP-analogue was determined by uv-measurements (1). Determination of free and immobilized enzyme activities was done spectrophotometrically as described previously (1). The assay system for hexokinase was as given by the Worthington Manual (7).

Activation with CNBr of Sepharose CL-4B was done according to March et al (8). Cyanate groups were determined by the method described by Kohn and Wilchek (9). Coupling of trypsin inhibitor (10 mg/g gel) and [¹²⁵I] albumin (5 mg/g gel) to CNBr and tressyl chloride activated Sepharose CL-4B were done at pH 7.5 and 4 °C as described above. Coupling yield and leakage of albumin were determined by measuring [¹²⁵I] activity of the gel and the collected washings.

Affinity experiments were done with the gels packed in Whatman columns of 0.3 cm diameter. The flow rate was controlled with a peristaltic pump. The absorbance of the eluate was recorded with a Uvicord (Pharmacia). The experiments were done at 4 °C except for the purification of peroxidase which was done at room temperature. Gels used for affinity chromatography were prepared by coupling at pH 7.5 and 4 °C.

Soybean trypsin inhibitor (15 mg) was coupled to 1.5 g Sepharose 4B (0.8 mmol tressyl groups/g dry weight) as described under Coupling. To remove any interfering tressyl groups, the coupled gel was washed with cold 0.1 M Tris-HCl, pH 7.5, for 30 min on a glass filter. A mixture of 4 mg albumin (bovine, type V, Sigma) 2 mg trypsin (bovine pancreas, type III, Sigma) and 2 mg α-chymotrypsin (bovine pancreas, type 1-S, Sigma) in 0.5 ml effluent (0.2 M sodium phosphate, pH 7.2) was applied to 1 g gel containing 7.5 mg inhibitor. The flow rate was 20 ml/h. Peroxidase (horse-radish, grade II, Boehringer) was applied to 0.5 g Sepharose 4B containing 6.5 mg Concanavalin A. The effluent contained 0.1 M sodium acetate pH 6.0, 1 M sodium chloride and 1 mM MgCl₂, MnCl₂, and CaCl₂ each. After application of 0.25 ml enzyme solution (containing 1.25 mg protein) the flow was stopped for 30 min to allow equilibration. Fractions were then collected at a flow rate of 10 ml/h.

Preparation of AMP-silica used for High Performance Liquid Affinity Chromatography (HPLAC). LiChrosorb[®] Diol (5 μm, Merck), 2.5 g dry weight, washed with acetone as described under Activation with tressyl chloride, was suspended in acetone (10 ml) and pyridine (1 ml) at room temperature. Tressyl chloride (0.5 ml) was added dropwise (1 min) with stirring. The gel was washed after 20 min as described under Activation. N⁶-(6-aminoethyl)-AMP (40 mg) was dissolved in 2.5 ml 0.7 M sodium bicarbonate and added to the wet tressyl silica (7 g). Coupling proceeded for 20 h at 20 °C at pH 7.5. After washing with coupling buffer the gel was treated with 0.1 M mercaptoethanol, pH 7.5, for 4 h at 20 °C (treatment with 0.1 M Tris-HCl buffer, pH 7.5, is believed to be a good alternative as was found for Sepharose bound affinity ligands). After washing of the gel as described under Coupling it was washed with acetone, sucked dry and analyzed for nitrogen. It was found that 13.2 mg AMP-analogue/g dry gel had coupled (i.e. 79 % yield).

Table 1. Activation of Sepharose with tresyl chloride.

Type of Sepharose	Amount of tresyl chloride used ^a mmol/g wet gel	Amount of tresyl groups formed ^b mmol/g dry weight
4B	0.15	0.80
4B	0.30	1.28
CL-4B	0.15	0.79
CL-4B	0.30	1.35
CL-6B	0.10	0.45
CL-6B	0.20	0.98
CL-6B	0.30	1.10

a) The reactions were done in acetone for 10 minutes as described in Materials and Methods.

b) Calculated from the sulphur content as determined by elemental analysis.

RESULTS AND DISCUSSION

Activation. The degree of substitution of various Sepharose preparations as a function of different amounts of tresyl chloride used during activation was studied (Table 1). As seen the same substitution pattern was obtained for the different Sepharose preparations. Noteworthy is the high amount of tresyl groups obtained after only 10 min of reaction (>1 mmol/g dry product). The swelling properties of Sepharose did not change during the activation since the settled volume of the gel was unchanged when transferred back to water.

The ratio of fluorine to sulphur, as determined by elemental analysis, corresponded well with the expected numbers. No nitrogen was introduced indicating that no pyridine was incorporated during the reaction. The amount of both S and F decreased by only a few per cent on storage at 4 °C overnight in 0.1 M phosphate, pH 7.5. This rather high stability contrasts with CNBr activated agarose where the half-life of cyanate ester groups in the aqueous phase at neutral pH is reported to be about 30 min (9). For storage it is recommended to keep the gels in 1 mM HCl at 4 °C as it has been found that the coupling capacity for enzymes did not decrease after storage for at least three months under these conditions. Alternatively, the gels can be lyophilized or kept in acetone.

Table 2. Coupling of enzymes and affinity ligands to different supports activated with tresyl chloride.

Support ^a	Ligand ^d	Bound ligand mg/g dry support	Yield %	Specific activity relative to soluble enzyme %
Sepharose 4B	Concanavalin A	360	97	
Sepharose 4B	Soybean trypsin inhibitor	195	68	
Sepharose CL-6B	Hexokinase	94	53	26
Sepharose CL-6B	Trypsin	124	70	33 ^e
Cellulose	"	61	87	56
Diol-silica ^b	"	24	80	63
Sepharose CL-6B	N ⁶ -(6-amino- hexyl)-AMP	87	32	
Cellulose	"	49	44	
Diol-silica ^b	"	18	36	
Glycophase- glass ^b	"	15	45	
Hydroxyethyl methacrylate ^c	"	10	12 ^c	

- a) Sepharose 4B and CL-6B and cellulose contained 1.28, 1.1 and 0.5 mmol tresyl groups/g dry weight, respectively. Activation of all polymers lasted for 10 minutes and 1.0 ml pyridine and 0.5 ml tresyl chloride were added to 17 g gel-suspension (i.e. support suspended in acetone, see Materials and Methods).
- b) 1,2-dihydroxy-3-propoxypropylsilyl-silica and Glycophase-GTM/CPG-40 were used.
- c) Spheron P 100,000 (this preparation has a low surface area, which may explain the relatively low coupling yield).
- d) Amount of ligand applied/g wet gel during coupling: 13 mg Concanavalin A; 10 mg soybean trypsin inhibitor; 10 mg trypsin; 10 mg hexokinase and 16 mg N⁶-(6-aminoethyl)-AMP. Concanavalin A and trypsin inhibitor were coupled for 15 h at 4 °C and at pH 7.5 and hexokinase at pH 7.0. The comparative studies involving trypsin and the AMP-analogue were carried out with the preparations coupled at pH 8.2. The affinity experiments described in the text however were made on gels coupled at pH 7.5.
- e) On coupling for shorter time (4 h) 55 % was bound but the specific activity was higher (50 %).

Different hydroxyl group carrying supports were conveniently activated with tresyl chloride followed by efficient coupling of enzymes or affinity ligands under mild conditions (Table 2).

Coupling of enzymes. The coupling of trypsin inhibitor under various conditions was studied. As seen from Table 3, high coupling yields (72 %) were obtained at relatively neutral conditions (pH 7.5). For hexokinase pH 7.0 was shown to be optimal whereas trypsin bound more efficiently at a somewhat higher pH (8.0, see also Table 2). The stability of the labile enzyme hexokinase was found to be very

Table 3. Coupling of soybean trypsin inhibitor to tresyl Sepharose 4B (1.28 mmol tresyl groups/g dry weight). Coupling was done at 4 °C.

Amount inhibitor added mg/g wet gel	Time for coupling h	pH	Bound inhibitor mg/g dry support	Yield %
10	15	6.5	70	25
10	15	7.5	195	68
10(20)	15	8.5	211(366)	74(64)
10	15	9.5	223	78
10	15	10.5	195	68
10	1.5	8.5	200	70

high when immobilized to tresyl agarose as no decrease in activity was detected after storage for one month at pH 7.0 and 4 °C, while such storage for the free enzyme in solution resulted in loss of all enzymic activity after a few days. The normal reaction in the immobilization of biomolecules to tresyl group carrying supports is believed to involve nucleophilic displacement at the carbon atom of the matrix. At alkaline conditions (pH > 8.5) it might be possible that substitution of the fluorine of the terminal CF₃ group with the biomolecule in question occurs as a side-reaction. Although immobilization of enzymes or affinity ligands carried out at these high pH values did not adversely affect their performance, to obtain a clearcut nucleophilic displacement, immobilization at pH-values 7-8 is recommended, which anyhow is the pH range of choice, notably for enzymes.

Affinity chromatography. The affinity properties of immobilized trypsin inhibitor were tested by applying a mixture of albumin, α -chymotrypsin and trypsin. On application at pH 7, albumin and inactive molecules of trypsin and α -chymotrypsin appeared in the void volume as one sharp peak. The bound active forms of α -chymotrypsin and trypsin could subsequently be completely eluted by changing to 0.2 M acetate of pH 4.8 and 3.0, respectively. The capacity of a preparation containing 11 mg inhibitor/g wet polymer was shown to be 9 mg trypsin per g of sorbent. The specific binding of the glycoprotein peroxidase (from horse-radish) to Conca-

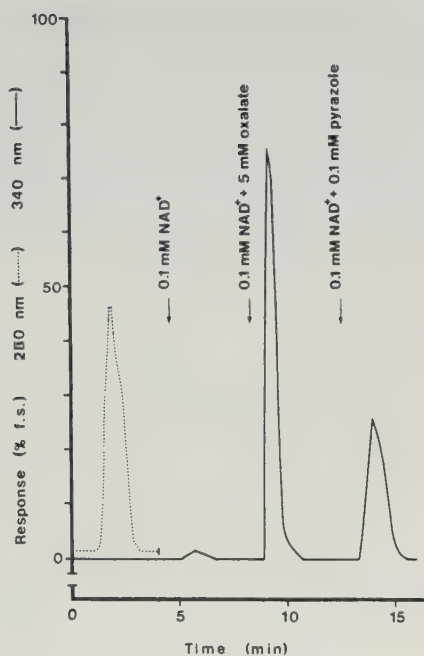


Figure 1. Separation of bovine serum albumin (25 μ g), beef heart lactate dehydrogenase (5 μ g) and horse liver alcohol dehydrogenase (25 μ g) on N^6 -(6-aminohexyl)-AMP-silica with HPLAC-technique. The AMP-analogue was coupled to tressyl chloride activated LiChrosorb Diol as described in Methods. The sample (450 μ l) was applied on a column (0.5x10 cm) containing 29 μ mol AMP-analogue/g dry support. The flow rate was 1 ml/min which gave a pressure drop over the column of 7 MPa. The column effluent (0.05 M sodium phosphate, pH 7.5) was monitored at 280 nm and then mixed on-line with assay reagents (13) for dehydrogenases and monitored at 340 nm. The experiment was done at room temperature.

navalin A immobilized on tressyl agarose was also demonstrated. On application of commercially available peroxidase the column was easily washed free of contaminating proteins or peptides and the bound peroxidase could completely be eluted by addition of 0.11 M mannose to the buffer. The obtained A_{403}/A_{280} ratio was 3.15, which corresponded well with the literature data for pure peroxidase (10).

To avoid possible non-specific binding to the gel, we have found that excess tressyl groups on the support can easily be removed by treating the affinity gel with 0.1 M Tris buffer, a strong nucleophile, at pH 7.5, 4 $^{\circ}$ C and for 30 min.

High Performance Liquid Affinity Chromatography (HPLAC). The results of a HPLAC experiment on N^6 -(6-aminohexyl)-AMP bound to diol-silica (LiChrosorb Diol) are shown in Figure 1. As seen the biospecificity of this general ligand is well conserved in analogy to previous findings with this ligand bound to CNBr activated Sepharose (11). No albumin was bound to the gel as 100 % appeared in the void volume and 80 % of added lactate dehydrogenase and alcohol dehydrogenase were recovered with short pulses of NAD^+ plus oxalate and NAD^+ plus pyrazole, respectively.

CONCLUSION

We have found tresyl chloride to be an excellent alternative to tosyl chloride for the activation of various hydroxyl group carrying supports, being better suited for the immobilization of pH-sensitive ligands and proteins. Activation of the support is rapid, leads to predictable and high degrees of substitution with tresyl groups and the activated gel is stable when stored even in aqueous media. In contrast, the widely applied CNBr activated supports rapidly loose their active groups in aqueous media. With regards to possible leakage it was found that on storage of $[^{125}I]$ albumin bound to tresyl chloride activated agarose (0.8 mmol tresyl groups/g dry weight) in 0.2 M Tris, pH 7.8 at 35 °C for 48 h, only a few per cent of the protein leaked off, whereas albumin bound to CNBr activated agarose (0.95 mmol cyanate groups/g dry weight) showed a loss of 20 %. This is of importance as leakage of proteins bound to CNBr activated gels in buffers containing amines or proteins is a well recognized problem (12). The coupling capacity at pH 7.5, 4 °C and overnight appears, as tested with albumin and trypsin inhibitor, to be higher for tresyl chloride than for CNBr treated gels containing the same amounts of active groups (82 and 70 % as compared to 55 and 60 %, respectively).

The activation method described is well suited for coupling of ligands and proteins used in affinity chromatography and should be especially useful for the preparation of immunosorbents as leakage of immobilized antibodies can be a major problem with the alternative techniques at hand. The ease of direct

activation of sorbents applicable to high performance liquid affinity chromatography constitutes an additional advantage.

ACKNOWLEDGEMENTS

We wish to thank the Swedish Natural Science Research Council for their support and Dr P.O. Larsson for helping us running the HPLAC experiment.

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IV

PEPTIDE SYNTHESIS IN AQUEOUS-ORGANIC SOLVENT MIXTURES WITH α -CHYMOTRYPSIN IMMOBILIZED TO TRESYL CHLORIDE ACTIVATED AGAROSE

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SUMMARY

α -Chymotrypsin was immobilized in high coupling yield (up to 80 %) to tresyl chloride activated Sepharose CL-4B. The immobilized enzyme was tested for its ability to synthesize soluble peptides from N-acetylated amino acid esters as acyl donors and amino acid amides as acceptor amines in water:water-miscible organic solvent mixtures.

It was found that the yield of peptide increased with increasing concentration of organic cosolvent. Almost complete synthesis (97 %) of Ac-Phe-Ala-NH₂ was obtained from Ac-Phe-OMe using a six-fold excess of Ala-NH₂. The rate of peptide formation in aqueous-organic solvent mixtures was good. Thus, 0.1 M peptide was formed in less than 2 h in 50 volume percent DMF with 0.1 mg immobilized chymotrypsin per ml reaction mixture.

The immobilized enzyme distinguished between the L and D configurations of acceptor amino acid amides even in high concentration of non-aqueous component (90 % 1,4-butanediol).

The effect of temperature was studied. It was found that both the yield of peptide and the stability of immobilized enzyme increased when the temperature was lowered. Experiments could be performed at subzero temperatures in the aqueous-organic solvent mixtures resulting in very high yield of peptide. After 3 weeks continuous operation at 4 °C in 50 % DMF the immobilized enzyme retained 66 % of its original synthetic activity.

The activity of the immobilized enzyme was better conserved with a preparation made from agarose with a higher tresyl group content as compared to a preparation

made from a lower activated agarose, indicating that multiple point of attachment has a favorable effect on the stability of the enzyme in aqueous-organic solvent mixtures.

The major advantage of using water-miscible instead of water-immiscible organic solvents to promote peptide syntheses appears to be the increased solubility of substrates and products, making continuous operation possible.

ABBREVIATIONS

DMF, N,N-dimethylformamide; Ac, acetyl; Ala-NH₂, alaninamide;
Gly-NH₂, glycinamide; Phe-OMe, phenylalanine methyl ester;
Phe-OEt, phenylalanine ethyl ester; Tyr-OEt, tyrosine ethyl ester.
Except when specified, the constituent amino acids were all of
the L-configuration.

INTRODUCTION

Already as early as 1937, Bergmann and Fraenkel-Conrat reported that proteases can catalyze peptide bond formation (1). However, it was not until a few years ago that more rigorous studies were published on the utilization of proteases for peptide syntheses on a preparative scale (for reviews see references 2 and 3). The greatest inherent advantages of enzymatic peptide syntheses over chemical are: (1) the risk for racemization is minimal; (2) minimal side chain protection is needed, especially when esters are used as acyl donors; (3) the proteases in most instances are stereospecific; (4) highly specific proteases can be used for fragment condensation. Furthermore, the possibility of utilizing proteases with a broader specificity (e.g. carboxypeptidase Y) to build up peptides step by step has been demonstrated (4).

Some drawbacks of enzymatic peptide syntheses are found, such as the cost of the enzyme, and the tendency of the enzyme to denature and aggregate in the aqueous-organic solvent mixtures which is often used to increase the yield of peptide.

In addition to preventing autolysis, immobilization of the enzyme should be advantageous if the enzyme can be reutilized many times even when used in the presence of aqueous-organic solvent mixtures; a prerequisite obviously being that the catalytic activity and specificity are preserved. Studies on immobilized proteases for this purpose are, however, still scarce and have dealt with addition of water-immiscible organic solvents and/or precipitation of the product in the reaction mixture to increase the yield (5-7). We therefore thought it worthwhile to investigate an alternative approach employing water:water-miscible organic solvent mixtures, as such systems would allow high

solubility of reagents and products, thus making possible continuous use of the immobilized enzyme.

In the present work, α -chymotrypsin was immobilized to various preparations of agarose, activated with different amounts of tresyl chloride. This was carried out with the hope to increase the enzyme stability in the aqueous-organic solvent mixtures, in analogy to literature reports on increased thermal stability of α -chymotrypsin by multiple point of attachment to supports (8). The synthetic activity, specificity and stability of the different preparations were compared as the concentration of organic solvents and the temperature were varied. Esters were used as acyl donors throughout (i.e. kinetic (nonequilibrium) approach), as they would allow rapid synthesis even in high concentrations of organic cosolvents and at low temperatures. It has been shown for many enzymes that at low temperatures (below 0 °C) they are stable, retaining their structure in high concentrations of organic cosolvent (9, 10).

MATERIALS AND METHODS

Sepharose CL-4B was from Pharmacia, Uppsala, Sweden. Glycophase glass (CPG-460) was from Pierce. 2,2,-trifluoroethanesulfonyl chloride (tresyl chloride) was from Fluka AG, Buchs, Switzerland. α -Chymotrypsin (bovine pancreas, type II, 38 U/mg, EC 3,4,21,1) was from Sigma, St. Louis, Mo., U.S.A. All amino acid derivatives were from Serva, Heidelberg, FRG, except D-Trp-OMe-HCl, which was from Sigma, St. Louis, Mo., U.S.A. Octadecyl-silica for reversed phase chromatography (Nucleosil^R 5 C₁₈) was from Macherey-Nagel Düren, FRG. The organic solvents (proanalysis quality) and molecular sieve, 4 Å, were purchased from Merck, Darmstadt, FRG.

Activation of agarose with tresyl chloride

Agarose was activated as previously described (11). The procedure to obtain the higher activation level was as follows: Sepharose CL-4B (10 g) was washed in succession with 100 ml of each of the following: 30:70 and 70:30 of acetone:water (V/V), acetone p.a. (twice) and three times with dry acetone (dried over molecular sieve, 4 Å). The gel was then transferred to a pre-dried beaker containing 3 ml dry acetone and 220 µl dry pyridine (dried with molecular sieve). During magnetic stirring, 220 µl tresyl chloride (Fluka AG, Buchs, Switzerland; also available from Fluorochem Ltd., UK) was added dropwise. After 10 min the gel was washed twice with 100 ml of each of the following: acetone p.a., 30:70, 50:50 and 70:30 of 5 mM HCl:acetone (V/V) and finally with 5 mM HCl and stored at 4 °C until used. The amount of introduced tresyl groups was determined on freeze-dried gel by elemental analysis of sulfur. The procedure to obtain the lower activation level was the same except that 45 µl tresyl chloride and 150 µl pyridine were used.

Coupling of chymotrypsin

Chymotrypsin (25 mg) was dissolved in 5 ml 0.2 M Hepes-HCl, pH 7.8, 4 °C, containing 5 mM Ac-D-Trp-OMe. The inhibitor was prepared from D-Trp-OMe after acetylation with acetic anhydride in glacial acetic acid (12). The solution was added to 5 g of activated gel, which had been rapidly washed with the above buffer. Coupling proceeded with gentle agitation for 12 h at 4 °C. The enzyme is expected to be bound in its monomeric form under the conditions used for coupling (13). The gels were rigorously washed with the following cold solutions: 0.1 M sodium acetate containing 0.5 M sodium chloride, pH 4; 0.5 M sodium chloride and distilled water. The remaining reactive tresyl groups were quenched by incubating the gels with 0.2 M Tris-HCl,

pH 8.5, for 2 h at room temperature. The gels were then washed in distilled water and finally with buffer for storage, 0.1 M sodium acetate, pH 4. The gels were stored at 4 °C. The amount of bound enzyme was determined by amino acid analysis of freeze-dried samples.

Peptide synthesis

Stock solutions of the nucleophile, Gly-NH₂, L- or D-Ala-NH₂, were made in water and the pH was corrected with 50 % NaOH-solution (Merck). The organic solvent was added to the desired concentration and the ester substrate was finally added. The solution (1 ml) was added to a plastic test tube (1.5 ml, Merck) containing 20-50 mg chymotrypsin-agarose. The test tube was gently agitated end over end. Samples (10 µl) were withdrawn at desired time points (10 min - 24 h) after a short centrifugation (30 s) of the test tube. The samples were added to test tubes (1.5 ml) containing 1 ml of eluent used in the HPLC-analyses of the samples (see below).

Experiments where product formation were followed every minute (see Fig. 2) were performed with the reaction solution magnetically stirred in a beaker. Chymotrypsin-agarose was added and samples (10 µl) were taken with a Gilson pipette through a nylon net (10 µm).

Syntheses in 90 % butanediol were carried out similarly, but the gel was washed twice with 20 gel volumes of each of 50:50 and 80:20 of butanediol:H₂O (V/V) before the gel was added to the reaction solution.

Determination of stability of immobilized chymotrypsin

The plastic test tubes with chymotrypsin-agarose and reaction solution were gently agitated for various amounts of time at 4, 22 or 37 °C, after which the gel was tested for its remaining activity. The supernatant was discarded after centrifugation and 1 ml of 30:70 of DMF:0.1 M NaHCO₃, pH 8.5, was added to wash the gel free of peptide and amino acid products. The test tube was gently agitated for some minutes, centrifuged and the supernatant discarded. The gel was washed twice more. Fresh substrate solution was added to the gel and the previous peptide synthesis experiment was repeated.

The stability in 80 % butanediol was determined by the same procedure, but using 50:50 of butanediol:0.1 M NaHCO₃ as washing medium.

HPLC-analyses

The samples were injected (200 µl) automatically on a HPLC-column (20 x 0.4 cm) filled with octadecyl-silica. The eluent, which consisted of 70:25:5 (V/V) water:DMF:glacial acetic acid, was pumped at room temperature through the column with a LDC HPLC-pump at a flow rate of 0.35 ml/min. The eluted substances were monitored spectrophotometrically at 270 nm.

Ester substrate, product acid and peptide were base-line separated in all cases. k' -values varied between 0.5 for peptide product and 3.0 for ethyl ester substrate. The yield of peptide was calculated from the peak areas. No correction for differing extinction coefficients was made.

RESULTS AND DISCUSSION

Coupling of α -chymotrypsin to tresyl chloride activated agarose

In Table I are shown activation and coupling data for the gels used in this study. The yield of bound enzyme was satisfactory in all cases. An inhibitor of chymotrypsin, Ac-D-Trp-OMe ($K_i = 9 \cdot 10^{-5}$ M (14)), was included during coupling to minimize the risk for autolysis of the enzyme. The activity per gram of gel for the synthesis of Ac-Phe-Gly-NH₂ from Ac-Phe-OMe and Gly-NH₂ was the same for all preparations in both 10 and 50 % DMF (N,N-dimethyl formamide), despite the fact that the amount of enzyme differed by about 25 % between the highest and lowest activated gels. The reason for this was not investigated but it could be that with the higher activation level the enzyme was less active because of a "tighter" binding to the support. This is in analogy to previous findings with α -chymotrypsin coupled to gels activated by high CNBr concentrations (8) and with horse liver alcohol dehydrogenase coupled in its ternary complex conformation to tresyl chloride activated glycerylpropyl-silica activated with high amounts of tresyl chloride (15).

Peptide syntheses with immobilized chymotrypsin

The mechanism for peptide syntheses by chymotrypsin has been extensively examined by Fastrez and Fersht (16). Schematically, the catalysis takes place in two steps: (1) the enzyme is acylated by a peptide, N-protected amino acid or ester with concomitant release of C-protected amino acid or peptide, water or alcohol; (2) a competitive deacylation between peptide or C-protected amino acid and water with the acyl-enzyme intermediate, which

yields peptide or hydrolyzed substrate (Scheme 1).

Scheme 1. Peptide synthesis catalyzed by chymotrypsin (CT-H) with Ac-Phe-X as acyl donor. HX denotes peptide, C-protected amino acid, alcohol or water and NH_2R denotes peptide or C-protected amino acid. In this study HX was HOCH_3 and NH_2R was $\text{NH}_2\text{CH}_2\text{CONH}_2$ or $\text{NH}_2\text{CH}_2(\text{CH}_3)\text{CONH}_2$.

While the rate of the deacylation step is similar with N-protected amino acids or their corresponding esters as acyl donors, there is a large difference in the acylation step, the ester reaction being several orders of magnitude more rapid.

The syntheses of peptides catalyzed by soluble chymotrypsin from N-protected amino acids (condensation or equilibrium method) or from the corresponding esters (nonequilibrium or kinetic approach) as acyl donors have been carefully compared (17, 18). It was found that esters are far more favorable allowing rapid (a few minutes) accumulation of product in reasonably high yields even with soluble products. The influence of water-miscible organic cosolvents on the peptide yield was not investigated. A colloidal dispersion of soluble chymotrypsin in 85 % 1,4-butanediol has been found to catalyze peptide bond formation from N-protected amino acids but the reaction was very slow (19). Several days were needed to obtain the optimal yield, probably due to the slow acylation process. Using esters as acyl donors more rapid reactions in aqueous-organic solvent mixtures are to be expected.

The results obtained using immobilized chymotrypsin in the formation of the peptide Ac-Phe-Gly- NH_2 from Ac-Phe-OMe and Gly- NH_2 in the presence of 10 and 50 %

DMF are shown in Fig. 1. As seen, the yield of peptide was higher in 50 % DMF than in 10 % DMF (70 % peptide compared with 30 %).

While the initial linear rates of product formation in 10 and 50 % DMF only differed by a factor of about 3, the time required to reach 100 % conversion of ester to products was about ten times longer with the higher DMF concentration. As discussed further below, the difference in rates is probably not caused by a decreased turnover number of the enzyme, but rather reflects a larger increase in K_m in 50 % DMF. However, even with a nonsaturating concentration of ester, the rate of peptide production was rapid in 50 % DMF (see Fig. 1). The reaction in 50 % DMF was about twice as rapid at a pH of 8.5 instead of 9.5.

A characteristic feature for the reaction in 10 % DMF was that the rate of acid formation was almost constant until the amount of soluble ester decreased below 15 mM, while the rate of peptide formation decreased gradually during the entire reaction as a consequence of the consumption of the acceptor amine (20).

The amount of peptide decreased only very slowly after all ester had been used up. As indicated above, this can be expected since the turnover number for the hydrolysis of peptides by chymotrypsin is orders of magnitude slower than for the hydrolysis of the corresponding esters (about 700 times difference in water media, pH 7.8 for Ac-Phe-OMe and Ac-Phe-Gly-NH₂ (20)). Furthermore, the K_m in aqueous media, pH 7.8, is about 20 times larger for the peptide than for the ester (21). The presence of Gly-NH₂ also decreases subsequent hydrolysis as does the lower water concentration in 50 % DMF. Subsequent hydrolysis of the product peptide is consequently very slow compared with its rate of synthesis. Thus, the amount of peptide decreased only with about 1 % when the solution was allowed to stand for an additional 5 h after all ester was consumed in 10 % DMF (i.e. after about 15 minutes). In 50 % DMF virtually no decrease was observed

15 h after complete ester conversion (i.e. after about 2 h). This further adds to the convenience of using ester substrates together with organic cosolvents for enzymatic peptide synthesis. It should be mentioned that no precipitation of the product was obtained in either 10 or 50 % DMF.

The influence of varying the substrate and its concentration

As seen in Table II the yield of peptide increased from 10 to 64 % when the concentration of Ac-Phe-OMe and Gly-NH₂ was increased from 25 mM to 0.5 M. Ester and products were completely soluble in 50 % DMF in this concentration range. The yield of peptide increased from 87 to 97 %, i.e. almost exclusively the peptide was formed, when the concentration of Ala-NH₂ was increased from 0.2 to 0.6 M.

The yield of peptide with ester substrates is determined by the relative reactivities between the acceptor amino acid amide and water with the acyl-chymotrypsin intermediate (Scheme 1). The nucleophilic potency of the different acceptor amines for deacylation of Ac-Phe-chymotrypsin has been measured in aqueous phase (16). It was found that 1 M Ala-NH₂ and Gly-NH₂ were 44 and 11 times, respectively, more reactive than 55 M water calculated from product ratios (aminolysis/hydrolysis) at pH 9.3. The product ratios that can be calculated from data given in Table II show that 0.6 M Ala-NH₂ and Gly-NH₂ are about 32 and 10 times more reactive against the acyl-enzyme than the water in 50 % DMF (the reactivities are slightly higher, since about 0.1 M of the acceptor amines is consumed during the reaction). The high amount of DMF or the immobilization therefore do not seem to have destroyed the ability of the enzyme's secondary binding site to distinguish between different acceptor amines. The stereospecificity of this site also seems to be largely unaffected as D-Ala-NH₂

was a much less potent nucleophile (about 25 times according to product ratios) than L-Ala-NH₂ (22).

The rate of product formation was practically unaffected when either Gly-NH₂ or Ala-NH₂ were increased from 0.2 to 0.6 M concentration with Ac-Phe-OMe as ester substrate in 50 % DMF. This shows that deacylation is not rate limiting with 0.1 M ester in 50 % DMF, since the rate then should increase with the concentration of acceptor nucleophile (15, 19). However, the rate increased almost linearly when the concentration of ester was increased from 25 to 500 mM. Although no separate K_m determination was made (due to difficulties in substrate solubility at higher concentrations), this indicates that the K_m for the ester substrate had increased drastically when the enzyme was exposed to 50 % DMF (the K_m for Ac-Phe-OMe of soluble chymotrypsin is reported to be 0.9 mM without organic cosolvent at pH 8 (21)).

In a more rigorous study on the effect of organic cosolvent, a drastic increase of K_m (about 1000 times) was observed for substrates of immobilized chymotrypsin in 50 % dioxane relative to water while the rates of acylation were practically unaffected (23). Similar results have been obtained with other cosolvents (24). Because of the constant acylation rates the increased K_m values were ascribed to a hydrophobic effect and not to distortion of enzyme conformation. Hydrophobic substrates partition more favorably to the bulk aqueous-organic solvent mixture than to the hydrophobic binding site of the enzyme, the higher the cosolvent concentration.

In aqueous media the rate of hydrolysis catalyzed by chymotrypsin is greater for Ac-Tyr-OEt as substrate than for Ac-Phe-OMe, which in turn is hydrolyzed more rapidly than Ac-Phe-OEt (21). Peptide syntheses with these esters catalyzed by immobilized chymotrypsin in 50 % DMF also gave differences in rates. Thus,

with the conditions given in Table II (i.e. nonsaturating amount of ester), the ratios for the initial reaction rates for Ac-Tyr-OEt, Ac-Phe-OMe and Ac-Phe-OEt were 1.8:1.3:1. However, as to be expected, the same yield of peptide was obtained with Ac-Phe-OMe as with Ac-Phe-OEt, indicating that a common intermediate (Ac-Phe-chymotrypsin) is formed during the catalysis (16, 20).

Attempts to quantify maximum rates of product formation in different concentrations of DMF were not made. However, the initial linear rate of product formation with 0.5 M Ac-Phe-OMe and 0.5 M Gly-NH₂ completely dissolved in 50 % DMF was about 50 % higher than in 10 % DMF (about 15 mM of the ester was soluble at this concentration of DMF). This shows that from at least a technical point of view, the immobilized enzyme was more efficient in 50 % DMF with the substrates used. Actually, the turnover number of soluble chymotrypsin for the hydrolysis of substrate has been shown to increase upon addition of DMF (16, 25; see also reference 23).

Influence of pH and DMF-concentration

In Table III is shown how the yield of peptide was affected by the pH and the amount of DMF. The product ratio (formed peptide/formed acid) in 50 % DMF was about twice that obtained in 10 % DMF, i.e. as may be expected from the decreased amount of water in 50 % DMF.

Increased yield of peptide was also obtained with immobilized chymotrypsin when the pH was increased (Table III). The increased yield up to pH 11.5 in 10 % DMF is, however, not easily explained, since the pK_a of Gly-NH₂ is 8.2 in water and this value is affected very little by 10 % DMF, as is the pH of the carbonate

buffer (26). Morihara and Oka did not obtain increased yields with soluble chymotrypsin under the same conditions above pH 9.5 (17). Despite that a high ionic strength buffer (0.2 M sodium carbonate) was used, an explanation might be that accumulation of the hydrolyzed ester causes a more acidic microenvironment for the immobilized enzyme. The yield of peptide also increased in 50 % DMF when the pH was increased from 8.5 to 9.5.

Under the conditions used in Table III, the rate of product formation decreased drastically in 50 % DMF when pH was increased above 8.5. Thus, at pH 9.5 the activity was half of that at pH 8.5 and it was practically lost at pH 10 (as mentioned in the Methods section, pH was determined before addition of the organic cosolvent. For a review on the effect of organic cosolvents on pK_a of different buffers see reference 26). In 10 % DMF only a 50 % reduction in the rate of product formation was observed when the pH was increased from 9 to 11.5.

The K_m of specific substrates towards soluble chymotrypsin is increased several-fold when the pH is increased from 9 to 10.5 (27). Maximum rates are, however, less affected. Maximum rates or K_m at different pH-values were not determined for the immobilized enzyme and therefore a comparison with the pH-sensitivity of the soluble enzyme can not be made with the present data.

Influence of temperature on peptide yield

In the only report we have found in the literature on the influence of temperature on enzymatic peptide syntheses, the yield of Bz-Ala-Val-OH from Bz-Ala-OMe and Val-OH obtained with soluble carboxypeptidase Y in water without organic cosolvent was studied (4). A linear increase of peptide yield of about 10 % was obtained in the temperature range 25 - 45 °C. The effect was explained to be caused by the

decreased pK_a of the acceptor amino acid with temperature. As seen in Fig. 2 we obtained the reverse, i.e. increased peptide yield with decreased temperature, with immobilized chymotrypsin. The effect was quite pronounced. Thus, the yield of Ac-Phe-Gly-NH₂ was about 67 % at 22 °C, but 86 % at - 10 °C. Interestingly, the magnitude of the effect was practically the same for Gly-NH₂ and Ala-NH₂ as acceptor nucleophiles and also very similar if we used either 10 or 50 % DMF or 80 % butanediol.

Obviously, the reaction of water and the acceptor amino acid amide with the Ac-Phe-chymotrypsin intermediate have different temperature dependencies, aminolysis being more favorable compared with hydrolysis the lower the temperature. It has been argued that Ala-NH₂ and Gly-NH₂ do not fit into the binding site until the transition state is reached for the reaction with Ac-Phe-chymotrypsin (20, 28). It is possible that the temperature effect is caused by a stronger binding of the acceptor amine relative to water in the transition state of the deacylation reaction at lowered temperature, thus favoring aminolysis. The effect was the same with chymotrypsin-agarose preparations obtained by either high or low activation with tresyl chloride.

Stability of the immobilized chymotrypsin preparations

The stability of chymotrypsin immobilized to agarose in 50 % DMF was dependent on the degree of activation with tresyl chloride (Fig. 3), indicating that many points of attachment to the support had a favorable effect on retention of the enzyme's conformation in organic solvent. Multipoint attachment has previously been shown to stabilize enzymes against inactivation due to unfolding caused by increased temperature (8) or denaturing agents (29).

The difference in stability between the preparations increased with higher temperatures in 50 % DMF. For example, while the difference in stability at 4 °C was very small, it was quite pronounced at room temperature in 50 % DMF (Fig. 3). At 37 °C, chymotrypsin bound to low activated agarose rapidly lost activity, only 0.03 % remaining after one day. Under similar conditions the remaining activity of chymotrypsin immobilized to highly activated agarose was more than ten times higher.

At 4 °C the preparations were remarkably stable in 50 % DMF. Thus, after three weeks about 65 % of the original synthetic activity remained. As can be seen in Fig. 3 most of the activity loss occurred during the first days, after which the enzyme was practically stable.

As shown above, the yield of peptide is dependent on the specificity of the enzyme for both the carboxyl and amine component (cf. Table II). Storage, even at 37 °C, did not affect the yields obtained, thus showing retention of enzyme specificity.

The temperature at which denaturation of protein structure occurs decrease with increasing concentration of water-miscible organic cosolvent (9, 10). However, spectroscopic studies indicate that the conformation of soluble chymotrypsin is affected very little if the temperature is kept below the denaturation temperature of the enzyme in the aqueous-organic solvent mixture (10). High stability of immobilized chymotrypsin can thus be expected at subzero temperatures even in high concentrations of organic cosolvent.

Limiting factors of using this approach for enzymatic peptide syntheses is of course the decreased catalytic rate and substrate solubility with decreased

temperature. The problem with lowered catalytic rate should be especially pronounced with chymotrypsin using N-protected amino acids as acyl donors. With ester substrates, however, we found peptide syntheses sufficiently rapid even at subzero temperatures. Thus, 0.1 M Ac-Phe-OMe was converted to products in 6 h with 0.12 mg immobilized chymotrypsin/ml reaction solution containing 0.2 M Gly-NH₂. The substrates and the products formed (Ac-Phe-OH and Ac-Phe-Gly-NH₂) were all kept soluble. The relation between the catalytic rates at -10, 4 and 20 °C with the above conditions was roughly 1:3:6.

Other cosolvents

Other solvents than DMF were tested in a preliminary experiment (Table IV). Acetone, acetonitrile and methanol were found to give almost the same increase in peptide yield as DMF, whereas 1,4-butanediol and glycerol had a very small effect (at 50 volume percent organic solvent). It has been argued that polyols at moderate concentrations (10 - 40 volume percent) are preferentially excluded from the protein domain (30, 31). Consequently, the immediate environment of the protein may be the same in mixed aqueous-polyol solution as in water, at least at moderate concentrations of polyol. Dipolar aprotic solvents, on the other hand, have been advocated to have a greater tendency to bind to proteins (preferentially hydrophobic side chains) and thus may lower the concentration of water around the protein more effectively than the polyols (32). This may explain why the polyols had to be used at a much higher concentration to be effective in increasing the peptide yield than the dipolar aprotics.

With acetonitrile a two-phase system was obtained, resulting in a slow rate of reaction, about 50 times slower than in water. Methanol gave a similar slow reaction, probably resulting from the reaction of methanol with the acyl-enzyme

intermediate, which gives the starting ester, thus inhibiting the reaction (20). When the dipolar aprotic solvents acetone or DMF were used at 75 % concentration and at room temperature a very slow enzymatic reaction resulted that completely stopped after about 10 % conversion of 0.1 M ester into products.

The dihydroxylic solvent 1,4-butanediol could, however, be used at up to 90 % concentration at room temperature, the immobilized enzyme converting substrates completely into products. This resulted in that similar yield of peptide could be obtained with this solvent as with DMF and acetone, but at a slower rate, the rate in 80 and 90 % butanediol being about three times and twenty times slower than in 50 % DMF. The immobilized enzyme could distinguish between L- and D-Ala-NH₂ even in 90 % butanediol (Table IV).

The stability of the enzyme was the same or slightly higher in 80 % butanediol than in 50 % DMF. In 90 % butanediol, the preparations obtained from the highest and lowest activated agarose (Table I) retained 15 and 10 %, respectively, of their original activities after four days at room temperature.

Drawbacks with polyol cosolvents are their high viscosity and low ability to dissolve hydrophobic substrates for chymotrypsin, limiting their usability at lowered temperatures.

CONCLUSION

We have shown that addition of water-miscible organic cosolvent increases the yield of peptide produced by immobilized chymotrypsin from ester substrates and amino acid amide acceptors. Practically complete conversion (97 %) of 0.1 M

Ac-Phe-Ala-NH₂ was obtained in 50 % DMF with a six-fold excess of Ala-NH₂ over ester in 2 h with 0.1 mg immobilized chymotrypsin/ml. The immobilized enzyme can distinguish between L- and D-Ala-NH₂ in at least 50 % DMF and 90 % butanediol, giving much higher yields of peptide with the L- than the D-form. Acetone or DMF are more efficient to promote peptide synthesis than glycerol or butanediol when used at the same concentration. This is consistent with the theory that water is less excluded from the enzyme domain in water-polyol mixtures than in water-dipolar aprotic solvent mixtures (31).

The enzyme activity is more stable when it is immobilized to a highly tresylated support than to a low tresylated support, indicating that a multipoint type of attachment has a stabilizing effect on the enzyme conformation in aqueous-organic solvent mixtures. This effect has also been observed in water:water-immiscible organic solvent systems (33). Immobilization of the enzyme also removes the problem of lowered solubility of the enzyme in aqueous-organic solvent mixtures as well as making reutilization possible. The choice of model peptides in this work was based on previous experiments with soluble chymotrypsin (17). Other N- or C-protecting groups that are easier to remove than CH₃CO or NH₂ can also be used (17, 34). Alternatively, the NH₂ group may be removed by reacting the product with carboxypeptidase Y (4).

The use of high concentrations of water-miscible organic solvents used here to increase the yield of peptide, appears to be advantageous over the method relying on the low solubility of product formed in water-rich media. Since the products were soluble with the high cosolvent concentrations used, the separation of products from the support were easy. Continuous use of the support is possible and the enzyme activity is expected to be better conserved since no harsh extraction procedure is needed. It has been claimed to be advantageous if the product is insoluble since subsequent hydrolysis of the product peptide would

then be minimized (17). With chymotrypsin the risk for secondary hydrolysis to occur, however, is low since with this enzyme the rate for syntheses from ester substrates is much higher than a possibly competing peptide hydrolysis. Peptide synthesis using ester substrates was even more convenient in the presence of organic cosolvents, since in this case secondary hydrolysis was very slow. Thus, as mentioned in the text, the yield of peptide obtained in 50 % DMF was almost not affected 15 h after completion of the reaction.

Compared with two-phase systems (water:water-immiscible organic solvent) higher rates of syntheses and less risk for clogging of the carrier pores with precipitated product are expected. The greatest advantage with two-phase systems has been advocated to be that very high concentration of organic solvent can be used with little effect on enzyme structure. We have, however, shown here that immobilized chymotrypsin retains its specificity in 50 % DMF and is very stable when used at 4 °C.

Peptide syntheses with chymotrypsin-agarose from ester substrates have been shown here to be rapid even with high concentrations of organic cosolvents. Using the free acid form of the carboxyl donating amino acid, high concentrations of enzyme and long equilibration times would be necessary (18, 19). Further, there would be the risk of side-reactions, such as polycondensations or reaction with side-chains of acyl donors; using ester substrates this is minimized because of their rapid reaction (2, 3).

Side effects of the use of organic cosolvent with immobilized chymotrypsin seem to be an increased K_m for substrates and a decreased stability, the latter leading to loss of activity at room temperature if too high a concentration is used. These limitations can be overcome by using ester substrates at high concentrations and performing the reaction at low temperature. With high

concentrations of substrates the rate was shown to be even higher in 50 % DMF than in 10 % DMF.

In addition to increasing the stability in organic solvents of the immobilized chymotrypsin, lowering of the temperature also resulted in considerably increased yields of peptide. The reaction of the immobilized enzyme with ester substrates was sufficiently rapid even at subzero temperatures.

We feel that the combination of immobilized thiol or serine proteases, ester substrates, low temperatures and high concentrations of organic cosolvents will find its applications in enzymatic peptide syntheses. We also think that immobilization of enzymes can be a way to facilitate cryoenzymological studies in aqueous-organic solvent mixtures, as such studies often are limited by low enzyme solubility (9).

ACKNOWLEDGEMENTS

This investigation has in part been supported by a grant from the National Swedish Board for Technical Development (grant no. 80-3595). The linguistic advise given by Drs. Staffan Birnbaum and Stephen Cliffe is gratefully acknowledged. We would also like to thank Drs. Lars Andersson and Björn Ekberg for valuable discussions.

SCHEME 1

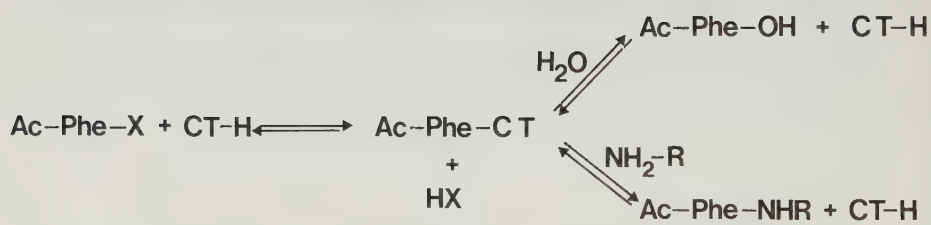


FIGURE LEGENDS

Fig. 1. Formation of Ac-Phe-Gly-NH₂ (●-●) and Ac-Phe-OH (▲-▲) from Ac-Phe-OMe (■-■) and Gly-NH₂ as a function of time in 10 % DMF (A) and 50 % DMF (B), respectively, catalyzed by chymotrypsin-agarose. The initial concentration of ester and acceptor amine were 0.15 and 0.2 M, respectively. (The solubility of the ester in 10 % DMF was about 15 mM). The pH was adjusted to 9.5 before addition of the organic solvent. Sodium bicarbonate (0.1 M) was used as buffer. The amount of wet gel was 50 mg per ml reaction solution.

Fig. 2. The influence of temperature on peptide yield with chymotrypsin-agarose. (■-■): 10 % DMF, 0.1 M Ac-Phe-OMe, 0.2 M Gly-NH₂; (●-●): 50 % DMF, 0.1 M Ac-Phe-OMe, 0.2 M Gly-NH₂; (▲-▲): 50 % DMF, 0.05 M Ac-Phe-OMe, 0.1 M Ala-NH₂; (□-□): 80 % butanediol, 0.05 M Ac-Phe-OMe, 0.1 M Ala-NH₂. The pH was 9.5 in 10 % DMF and 8.4 in 50 % DMF and 80 % butanediol (adjusted before addition of organic solvent).

Fig. 3. The influence of temperature and degree of tresyl chloride activation of agarose on the stability of chymotrypsin-agarose as tested by repeated assays in 50 % DMF with 0.1 M Ac-Phe-OMe and 0.2 M Gly-NH₂. The activity was calculated from the time to reach 15 % conversion of ester. (o-o): 4 °C, low-activated agarose; (●-●): 4 °C, high-activated agarose; (□-□): 22 °C, low-activated agarose; (■-■): 22 °C, high-activated agarose. The pH was 8.4 before addition of the organic solvent. No inorganic buffer was used. The amount of wet gel was 40 mg per ml reaction solution. The gel was stored in the reaction solution until the assay was repeated (see Methods).

FIGURE 1

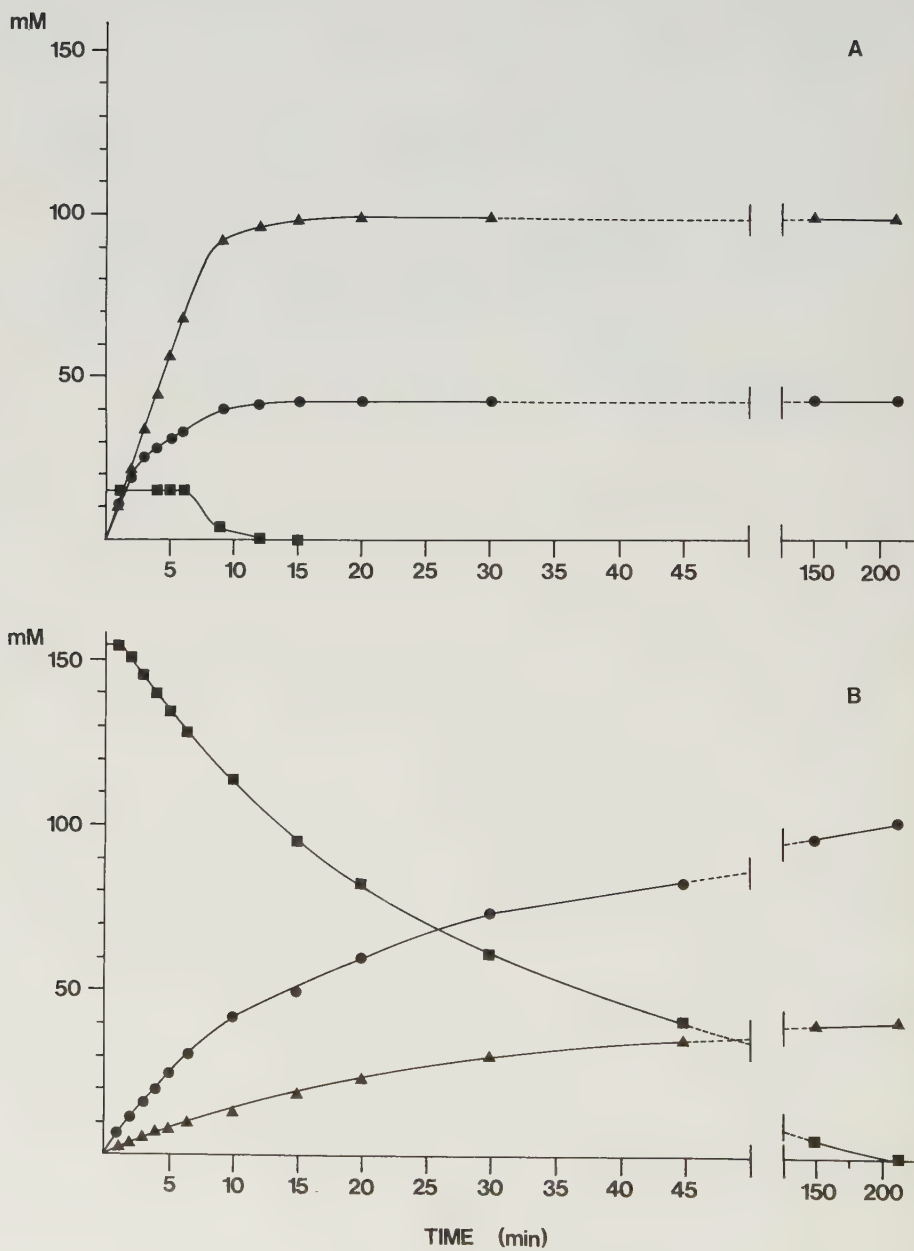


FIGURE 2

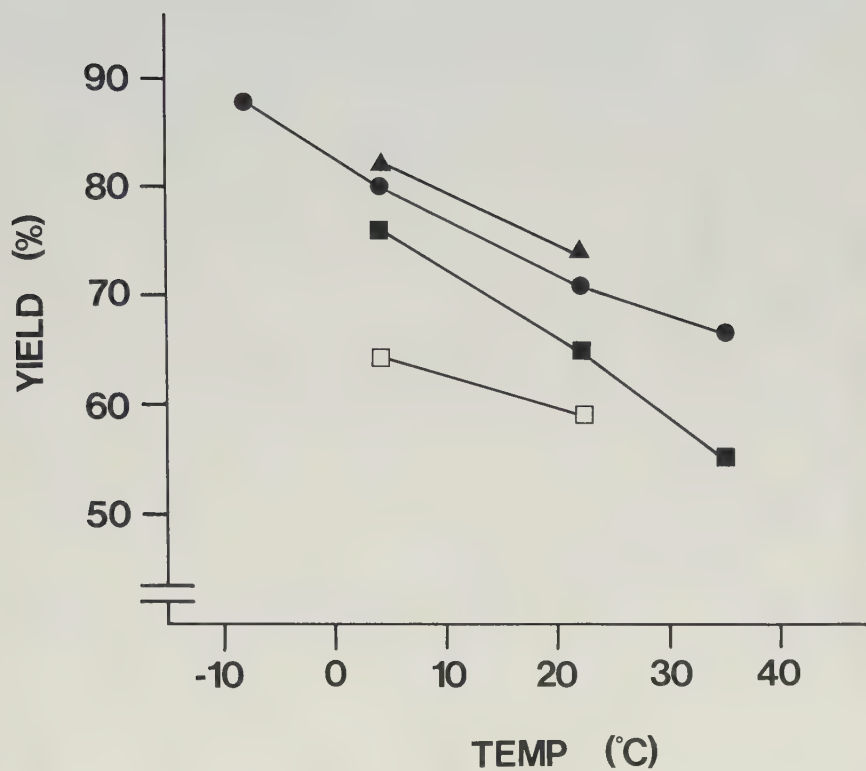


FIGURE 3

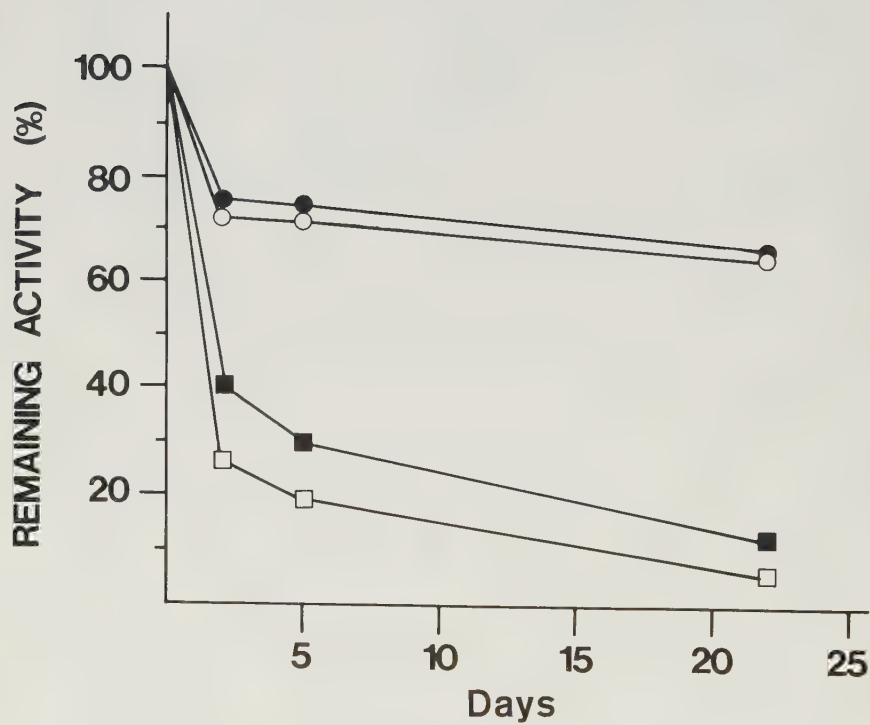


TABLE I.

Immobilization of chymotrypsin to tresyl-agarose.

Amount of tresyl chloride during activation mmol/g dry gel	Amount tresyl groups intro- duced mmol/g dry gel	Bound chymo- trypsin mg/g dry gel	Coupling yield %	Activity ^{a)} mmol/min, g dry gel
1.2	0.22	70	60	13.5
3.4	0.57	78	68	12.9
5.8	0.97	79	78	11.1

a) Calculated from the initial linear conversion of 0.1 M Ac-Phe-OMe into the products Ac-Phe-Gly-NH₂ and Ac-Phe-OH in the presence of 0.2 M Gly-NH₂ in 0.2 M NaHCO₃, pH 9.

TABLE II.

Yields of peptides obtained in 50 % DMF with chymotrypsin-agarose.

Donor ester	Concentration M	Acceptor nucleophile	Concentration M	Yield ^{a)} %
Ac-Phe-OMe	0.025	Gly-NH ₂	0.025	10
Ac-Phe-OMe	0.075	Gly-NH ₂	0.1	37
Ac-Phe-OMe	0.1	Gly-NH ₂	0.1	34
Ac-Phe-OMe	0.25	Gly-NH ₂	0.25	55
Ac-Phe-OMe	0.5	Gly-NH ₂	0.5	65
Ac-Phe-OMe	0.1	Gly-NH ₂	0.2	69
Ac-Phe-OMe	0.1	Gly-NH ₂	0.6	91
Ac-Phe-OMe	0.1	Ala-NH ₂	0.2	88
Ac-Phe-OMe	0.1	Ala-NH ₂	0.6	97
Ac-Phe-OMe	0.1	D-Ala-NH ₂	0.2	21
Ac-Phe-OEt	0.1	Gly-NH ₂	0.2	69
Ac-Tyr-OEt	0.1	Gly-NH ₂	0.2	65

a) Peptide in percent formed after complete conversion of ester substrate into products (cf. Fig. 1).

TABLE III.

Dependence on pH and concentration of DMF for the yield of Ac-Phe-Gly-NH₂ from Ac-Phe-OMe and Gly-NH₂ obtained with chymotrypsin-agarose^{a)}.

DMF %	pH	Yield %
10	9	14
10	10	19
10	11	25
30	9	19
50 ^{b)}	9	37

a) The concentration of Ac-Phe-OMe was 0.075 M and that of Gly-NH₂ was 0.1 M.

b) The activity of chymotrypsin-agarose in 50 % DMF was very low above pH 9 (see text).

TABLE IV.

Effects of different organic cosolvents on chymotrypsin-agarose catalyzed peptide syntheses^{a)}.

Solvent	Concentration %	Acceptor amine	Concentration M	Yield %
Acetone	50	Ala-NH ₂	0.2	84
Acetone	50	Gly-NH ₂	0.2	68
Acetonitrile	50	Gly-NH ₂	0.2	67
Methanol	50	Gly-NH ₂	0.2	68
Glycerol	50	Gly-NH ₂	0.2	40
Butanediol	50	Gly-NH ₂	0.2	40
Butanediol	50	Ala-NH ₂	0.1	67
Butanediol	80	Ala-NH ₂	0.1	80
Butanediol	90	Ala-NH ₂	0.1	88
Butanediol	90	D-Ala-NH ₂	0.1	33
Butanediol	80	Gly-NH ₂	0.2	65
Butanediol	90	Gly-NH ₂	0.2	86

a) Ac-Phe-OMe was used as donor ester in all cases. All the experiments with Ala-NH₂ in butanediol were done with 0.05 M ester and all others with 0.1 M ester concentration.

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High-Performance Liquid Affinity Chromatography on Silica-Bound Alcohol Dehydrogenase

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Received March 14, 1983

Horse liver alcohol dehydrogenase was immobilized on glycerylpropyl-silica (10 μm , 1000-Å pores) activated with 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride). The coupling and activity yield was almost 100%. The coenzyme-binding sites were equivalent and virtually unaffected by the immobilization process, as judged from Scatchard plots and active-site titrations. The silica-bound enzyme, packed in steel columns, was integrated with HPLC equipment and then successfully used for chromatography of adenine nucleosides, adenine nucleotides, and triazine dyes. Dissociation constants were calculated from chromatographic data and found to correspond well with literature values. The dissociation constants for a number of nucleotide derivatives with potential application in affinity chromatography were also determined. The spacers were found to affect the binding strength of the nucleotides in a qualitatively predictable way. Theoretical plate heights were calculated and found to be in the range 0.01 to 0.1 cm. Attempts to correlate peak widths with the rate constants for the binary complexes involved were only partially successful.

KEY WORDS: HPLC; affinity chromatography; high-performance liquid affinity chromatography; nucleotide separation; quantitative affinity chromatography; immobilization.

Most affinity chromatographic separations to date employ low-molecular-weight ligands, such as inhibitors, for the purification of high-molecular-weight compounds, such as enzymes. The complementarity of an enzyme and its inhibitor should endorse the reverse policy as well, i.e., to use a support-bound enzyme as a "ligand" for the separation of small molecules. However, this approach has not been pursued to any greater extent for a number of reasons. From a preparative point of view the approach is less attractive since the adsorbent is usually very expensive and, at least on a weight basis, has a very limited binding capacity. Even analytically, less attractive features are obvious. The very low capacity of the system may render detection difficult or imprecise.

In the present study we have tried to circumvent some of the obstacles hindering the use of enzymes as adsorbents for low-molecular-weight compounds by combining the

concept of affinity chromatography with the technique of high-performance liquid chromatography. Horse liver alcohol dehydrogenase (HLADH)¹ was thus coupled to silica by the efficient tresyl chloride-coupling method (1) and used for the separation of a number of substances. Chromatographic data that were obtained were used for determination of equilibrium and rate constants. This combination of techniques, which has been referred to as high-performance liquid affinity chromatography (HPLAC), has earlier been demonstrated to efficiently separate plasma proteins, enzymes, carbohydrates, and other biomolecules. The adsorbents involved were silica-bound ligands such as inhibitors (2,3), triazine

¹ Abbreviations used: Tresyl chloride, 2,2,2-trifluoroethanesulfonyl chloride; HLADH-silica, silica-bound horse liver alcohol dehydrogenase (EC 1.1.1.1); diol-silica, silica coated with a glycerylpropyl layer; STI, soybean trypsin inhibitor; HETP, height of one theoretical plate; HPLAC, high-performance liquid affinity chromatography.

dyes (4), serum albumin (5), boronic acid (6), antibodies (2,7), and concanavalin A (8).

MATERIALS AND METHODS

Materials. Silica, LiChrospher Si 100, LiChrospher Si 300, and LiChrospher Si 1000 were purchased from E. Merck, Darmstadt, FRG. Tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) was obtained from Fluka AG, Buchs, Switzerland. Horse liver alcohol dehydrogenase, NAD (free acid, grade 1), NADH (disodium salt, grade 1), AMP (disodium salt, crystallized), and 1,4-dithioerythritol (crystallized) were from Boehringer-Mannheim, Mannheim, FRG. Adenosine 5'-diphosphate (ADP, di-monocyclohexylammonium salt, grade IV), adenosine 5'-diphosphoribose (ADP-ribose, sodium salt), soybean trypsin inhibitor (type 1-S), and Cibacron Blue F3G-A (Reactive Blue 2) were from Sigma, St. Louis, Missouri. Procion Red H-3B and Procion Yellow MX-8G were obtained from ICI, England, and [*carbonyl*- ^{14}C]NAD (ammonium salt in 2% ethanol) was from Amersham International Ltd., Buckinghamshire, England. γ -Glycidioxypropyltrimethoxysilane (Z-6040) was obtained from Dow Chemicals, Midland, Michigan.

Nucleotide derivatives were prepared analogously to earlier-described procedures (9). All other chemicals used were of reagent-grade quality and double-distilled water was used throughout.

Preparation of diol-silica. Glycerylpropyl-silica, or "diol-silica," is now also commercially available from E. Merck, FRG, in 100-Å pore size. The procedure for its preparation is outlined below.

Silica (LiChrospher Si 100, LiChrospher Si 300, or LiChrospher Si 1000), 20 g, was placed in a three-necked reaction flask, where it was dried for 4 h at 150°C under vacuum. The reaction flask was then cooled and sodium-dried toluene (300 ml) was added, followed by 20 ml of γ -glycidioxypropyltrimethoxysilane and 0.5 ml KOH-dried trimethylamine. The suspension was stirred with an

overhead stirrer and refluxed at normal pressure for 16 h. To ensure anhydrous conditions, the reflux condenser was fitted with a calcium chloride tube. The resulting epoxy-silica was washed on a glass filter with toluene, acetone, and ether and subsequently dried under vacuum. The epoxy-group content was determined by titration of a sample with HCl after addition of sodium thiosulfate (10). The epoxy-group content was 2.5 $\mu\text{mol}/\text{m}^2$ of the nominal surface area.

The diol-silica was finally obtained by heating epoxy-silica at 90°C in 0.01 M HCl for 2 h (50 ml HCl/g epoxy-silica). The finished product was washed with water and acetone and vacuum dried.

Preparation of tresyl-silica. Diol-silica was activated according to Nilsson and Mosbach (1). Two grams of diol-silica (1000-Å pore size) was washed with 3×50 ml dry acetone (treated with molecular sieve, 4 Å). The wet silica (5 g) was transferred to a beaker containing 2.5 ml dry acetone and 200 μl dry pyridine. The suspension was cooled in 0°C on an ice bath and the activation was initiated by the dropwise addition of 65 μl tresyl chloride during vigorous magnetic stirring. After 20 min the gel was washed with two 50-ml vol of each of the following: 100% acetone, 25:75, 50:50, and 75:25 of 5 mM HCl:acetone (v/v), and 100% 5 mM HCl. The activated gel was either used directly for enzyme coupling or washed with acetone, freeze-dried, and stored in a desiccator until required. The degree of activation was determined by the elemental analysis of sulfur.

Coupling of HLADH to silica. Six milliliters of the commercial HLADH preparation (10 mg/ml) was centrifuged, the supernatant was discarded, and the precipitate was resuspended in 5 ml 0.075 M sodium phosphate buffer, pH 7.9. The HLADH was then dialyzed against the same buffer (2 liters) for 1.5 h at room temperature. The dialysate was centrifuged and the HLADH concentration in the clear solution was determined spectrophotometrically ($A_{280}^{1\% \text{ cm}} = 4.5$).

Tresyl-silica (1000 Å; 10 μm ; 0.71 g dry wt)

was suspended in 2 ml cold 0.4 M sodium phosphate buffer, pH 7.0, containing 2 mM NADH and 0.2 M isobutyramide. The suspension was deaerated in a vacuum and 2 ml of the dialysate containing 15 mg of alcohol dehydrogenase was added. Coupling proceeded for 20 h at pH 8.0 and room temperature. Any remaining tresyl groups were subsequently removed by treatment with 0.2 M Tris-HCl + 1 mM dithioerythritol, pH 8.0, for 1 h. The gel was then washed extensively with 0.5 M NaCl + 1 mM dithioerythritol in 0.1 M sodium phosphate buffer, pH 7.5, followed by 1 mM dithioerythritol, pH 7.5, in 0.15 M sodium phosphate buffer. The horse liver alcohol dehydrogenase-silica (HLADH-silica) was stored at 4°C until used.

Packing of columns: Determination of column contents. Stainless-steel columns (50 × 5 mm) with compression fittings were used (hetp, Macclesfield, U.K.). HLADH-silica (~0.7 g) was suspended in 0.25 M sodium phosphate buffer, pH 7.5, containing 1 μ M ZnSO₄ and 25% sucrose. The suspension was treated for 5 min in an ultrasonic bath and then immediately poured into the packing device. The packing device was made up from a 25-cm, 1/4-in stainless-steel tube attached at one end to the column to be packed via a bored-through union. The other end was connected to an Altex HPLC pump delivering 0.25 M sodium phosphate buffer, pH 7.5, containing 0.1 μ M ZnSO₄ at a pressure of 3000 psi. After 30 min of pumping, the packing was considered complete. Columns were stored at 4°C.

A 5-cm column has an inner volume of 1.0 ml. By weighing empty columns, filled columns, and freeze-dried columns it was determined that a column contains 0.44 g silica (1000-Å pores) adsorbent (dry weight) and that it has a liquid-phase volume of 0.80 ml (i.e., pore volume + interstitial volume). The concentration of binding sites in a column was calculated on the liquid-phase volume.

Chromatographic procedure. The chromatographic runs were undertaken at ambient temperature (20–24°C). The eluant was 0.25

M sodium phosphate buffer, pH 7.5, containing 1 μ M ZnSO₄ unless otherwise stated. All buffers were prepared from water of glass-distilled quality. The chromatographic setup consisted of an Altex Model 110 HPLC pump (Altex Scientific, Berkeley, Calif.), a valve system, an affinity column, a uv/vis detector (LDC Spectromonitor III, Riviera Beach, Fla.), and a recorder. The valve system consisted of two Valco 7000 six-port valves (Valco Instruments, Houston, Texas). The sample (15 μ l) was introduced via the first valve. The second valve allowed a convenient switching of the flow between the column and a bypass loop. The arrangement is greatly appreciated when recovery studies and saturation studies are undertaken.

RESULTS AND DISCUSSION

High-performance liquid affinity chromatography is the advantageous combination of affinity chromatography and HPLC (10). Because the present study utilized a delicate enzyme, liver alcohol dehydrogenase, as a ligand, extra precautions were necessary when choosing the support and coupling procedure, as well as the chromatographic conditions. In this way it was possible to fully realize the separation potential of the ligand and to ensure that the separation pattern truly reflected the molecular properties of the reversible complex between HLADH and chromatographed compounds, thus allowing meaningful calculations of equilibrium constants and rate constants.

Properties of Tresyl-Silica

Figure 1 gives an overview of coupling with the tresyl chloride method. The method has been previously utilized for the coupling of a number of compounds to various hydroxyl-containing supports, such as agarose and silica (1). However, the coupling of high-molecular-weight compounds such as proteins to silica has not been investigated and we felt such a study appropriate. For economic reasons soybean trypsin inhibitor (STI) was used initially.

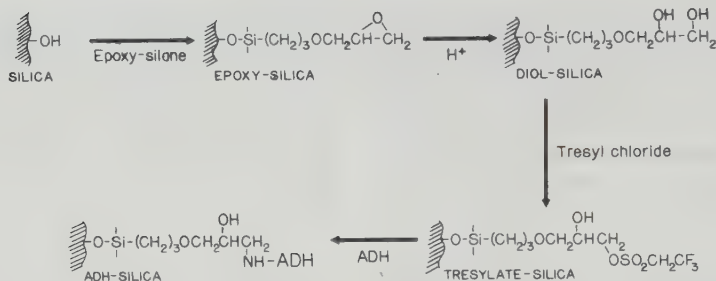


FIG. 1. Preparation of HLADH-silica via tresyl chloride-activated glycerylpropyl-silica (diol-silica).

The amount of tresyl groups introduced on diol-silica was easily controlled by varying the amount of tresyl chloride during the activation procedure (Table 1). As seen in the table, a 300-Å diol-silica was substituted with 50, 125, or 400 μmol tresyl groups per gram of silica, which corresponds to 12, 30, and 100% diol-group substitution.

Table 1 also shows that short coupling times may suffice with tresyl-silica. For instance, when using medium or highly activated 300-Å silica, almost 80% of the STI was bound

after 1 h compared with the value obtained after 20 h. As expected STI could be coupled in high amounts to tresyl-activated silica. The observed coupling (about 50 mg/g) for 1000-Å silica thus corresponds to about 2.5 mg protein/ m^2 of silica surface which means a close-packed binding pattern (10). The slightly lower value calculated for 100-Å silica—2 mg/ m^2 —may be due to steric crowding, since in this case pore and ligand diameters are of a more comparable size.

Summarizing, the tresyl chloride method

TABLE 1
COUPLING OF SOYBEAN TRYPSIN INHIBITOR TO TRESYL CHLORIDE-ACTIVATED DIOL-SILICA

Pore size (Å)	Amount of tresyl chloride added ^a ($\mu\text{mol/g}$ dry silica)	Amount of tresyl groups introduced ($\mu\text{mol/g}$ dry silica)	Amount of STI added ^b (mg/g dry silica)	Amount of STI bound (mg/g dry silica) after	
				1 h	20 h
100	2900	450	300	n.d.	250
100	2900	450	900	n.d.	250
300	160	50	350	42	103
300	410	125	350	90	115
300	2450	400	350	220	279
1000	1800	60	50	n.d.	46
1000	1800	60	200	n.d.	54

^a The activation procedure is described under Materials and Methods. The amounts of pyridine used were 300 and 200 $\mu\text{l/g}$ dry diol-silica 100 and 1000 Å, respectively. The amount of pyridine used when activating the three samples of 300-Å diol-silica was 100, 100, and 300 $\mu\text{l/g}$ dry diol-silica, respectively.

^b Coupling procedure: Tresyl-silica (100 mg dry wt) was suspended in 1.3 ml cold coupling buffer (0.3 M sodium phosphate + 0.3 M sodium chloride, pH 8.0) containing STI. Coupling proceeded at 4°C. The gels were washed as described in Ref. (1).

appears to be suitable for the coupling of sensitive ligands to silica as it is rapid and can be performed under mild conditions.

Coupling of HLADH to Silica

As discussed below, chromatographic data obtained with HLADH-silica were treated quantitatively to allow convenient calculation of involved binding constants. A fundamental question in this context is whether the immobilized enzyme has the same binding properties as the free one. The binding properties could be envisaged to be altered due to microenvironmental factors contributed by the support, by steric hindrance, by covalent modification, and by loss of the catalytic zinc. Several precautions were therefore observed during the coupling process to preserve the native character of the enzyme as much as possible. The status of the bound enzyme was subsequently assessed in a number of tests.

To facilitate unhindered interaction between enzyme and chromatographed substances, large-pore silica (1000-Å pores) was used throughout. Based on the experiments with STI, low- and medium-activated silica was considered most suitable when coupling

alcohol dehydrogenase. A low activation level should minimize the possibility of "freezing" the enzyme in any particular conformation (by introducing many points of attachment), thereby changing its normal binding properties (11). Furthermore, by coupling the enzyme as a ternary complex with NADH and isobutyramide, unwanted modification of the nucleotide-binding site, e.g., of lysine 228 (12,13), and loss of the catalytic zinc (14,15) should be prevented.

As shown in Table 2, the coupling yield for HLADH was 100% in the case of medium-activated silica (1000 Å), while the low-activated silica bound 57% of added enzyme. The dissociation constant (0.8–0.9 μM) obtained for the complex NADH-immobilized enzyme is very close to that reported for the soluble enzyme (0.6 μM (16), 1.2 μM (14)). The almost quantitative retention of activity for HLADH-silica was an indication that the small particle size (10 μm) minimized diffusional hindrances in the assay. The retained activity further indicated that the rate constants of enzyme-ligand complexes are unaltered by immobilization, since it is known that the dissociation of the coenzyme (NADH) is the rate-limiting step in alcohol dehydrogenase catalysis. The

TABLE 2

COUPLING OF HORSE LIVER ALCOHOL DEHYDROGENASE TO GLYCERYLPROPYL-SILICA:
PROPERTIES OF ALCOHOL DEHYDROGENASE-SILICA

Amount of tresyl chloride used in the activation (μmol/g dry silica)	Amount of enzyme added (mg/g dry silica)	Amount of enzyme bound (mg/g dry silica)	Relative activity of bound enzyme compared with free enzyme (%)	Retention of coenzyme binding sites (%)	Dissociation constant NADH (μM)
100	21	12	100	100	0.87
300	21	21	95	97	0.80

Note. Activation of diol-silica (1000 Å; 10 μM) was carried out as described under Materials and Methods. The HLADH activity was measured at 340 nm (reduction of NAD). The assay mixture contained 9 mM NAD and 10 mM ethanol in 0.25 M sodium phosphate buffer, pH 7.8. The assay of silica-bound enzyme was carried out in a magnetically stirred cuvette (1.50 mg silica in 2.5 ml assay mixture). The coenzyme-binding sites were determined by ternary complex formation. To this end HLADH-silica was incubated with ^{14}C -labeled NADH (10 μM) + 100 μM isobutyramide in 0.25 M sodium phosphate buffer, pH 7.6. After 15 min the silica was spun down and the radioactivity of the supernatant measured. The dissociation constant for NADH without isobutyramide was determined in a Scatchard plot (Fig. 2).

intrinsic dissociation rate constants could thus be expected to be the same, at least when they are of the same magnitude as the one describing the dissociation of NADH.

Furthermore, the linear Scatchard plots shown in Fig. 2 indicated that the immobilized enzyme population is largely homogeneous with respect to coenzyme-binding ability (17,18). The concentration of binding sites calculated from the Scatchard plot was slightly lower (less than 10%) than an independent active-site determination with NADH + isobutyramide. Since active-site determination is based on ternary complex formation, which more easily detects very weak binding sites, the discrepancy between the two methods may indicate the presence of a small population of enzyme having a weak binding site.

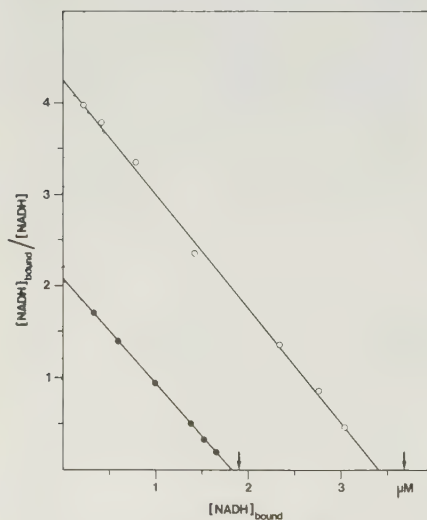


FIG. 2. Scatchard plot for NADH binding to HLADH-silica. Alcohol dehydrogenase silica (1000 Å) containing 12 mg enzyme/g silica (●) or 21 mg enzyme/g silica (○) was equilibrated for 15 min at 20°C with a solution containing ^{14}C -labeled NADH and varying amounts of unlabeled NADH in 0.25 M sodium phosphate buffer, pH 7.6. The silica was subsequently centrifuged down and the activity of the supernatant measured. The arrows show the amount of NADH bound in an independent determination assay in which the adsorbents were saturated with NADH + isobutyramide (Table 2).

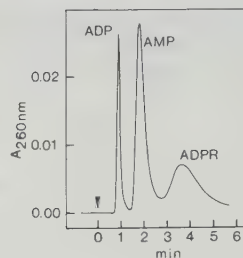


FIG. 3. Separation of adenine nucleotides on HLADH-silica (1000 Å; 10 μm). The binding-site concentration was 160 μM. Sample concentrations: 30 μM AMP, 15 μM ADP, and 60 μM ADP-ribose. Flow rate: 1 ml/min.

Summarizing, carefully prepared HLADH-silica appears to contain a largely homogeneous population of binding sites with properties very similar to those of free enzyme.

High-Performance Liquid Affinity Chromatography

Figure 3 shows the separation profile of a mixture of the adenine nucleotides AMP, ADP, and ADP-ribose on an HLADH-silica column. As expected from the previous knowledge of the interaction between alcohol dehydrogenase and these compounds, ADP was eluted first, followed by AMP and then ADP-ribose. A convenient measure of the retention of a compound is given by its k' value (mass distribution ratio; column capacity ratio (19)) calculated by

$$k' = (T_e - T_0)/T_0 \quad [1]$$

where T_e is the elution time for the compound in question and T_0 is the elution time for a nonexcluded and nonadsorbed compound. The k' value thus refers to the number of column volumes a substance is retained.

As seen in Fig. 3, ADP is very weakly retained— $k' = 0.10$. However, when a column with a higher enzyme content was used, proportionally higher k' values were obtained. For example, k'_{ADP} increased to 0.19 when the enzyme content increased from 12 to 21 mg/g silica. To achieve a more substantial increase

of k'_{ADP} a higher enzyme loading than is possible with 1000-Å silica would be necessary.

Figure 4 shows the separation of a mixture of closely related AMP analogs on HLADH-silica. A minor impurity could also be detected (peak b), stemming from the N^6 -(2-aminoethyl)-AMP preparation used. Impurities in commercially obtained AMP and ADP-ribose could also be detected when these compounds were separately chromatographed on HLADH-silica (k' values for the impurities corresponded with those of ADP and AMP).

Determination of dissociation constants. A simple relationship between k' , the mass distribution ratio, and the dissociation constant (K_d) of the complex (ES) between a silica-bound enzyme binding site (E) and a solute (S) can be derived (20–23) as

$$k' = \frac{[\text{bound solute}]}{[\text{free solute}]} = \frac{[\text{ES}]}{[\text{S}]} \quad [2]$$

and

$$K_d = \frac{[\text{E}][\text{S}]}{[\text{ES}]} \quad [3]$$

Combining Eq. [2] and Eq. [3] gives

$$k' = \frac{[\text{E}]}{K_d} \quad [4]$$

If the sample introduced on the column is much smaller than the amount of binding sites, Eq. [4] may be written as

$$k' = \frac{e_0}{K_d} \quad [5]$$

where e_0 is the total concentration of binding sites. The relationship (Eq. [5]) should be useful for predicting the behavior of affinity chromatography systems. Conversely, unknown dissociation constants could be easily calculated from a chromatogram. The validity of the approach obviously requires that the effective ligand concentration is known and that the complexes formed close to the silica surface are the same as the corresponding free ones. When a small ligand, such as an inhibitor, is bound to silica in concentrations in the millimolar range, it is easily conceivable that a substantial proportion will be located so as to

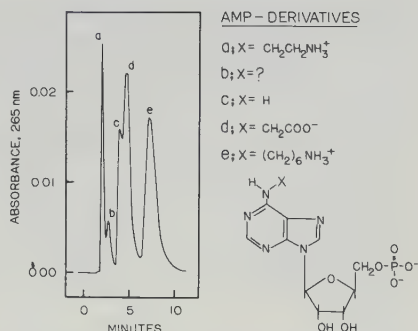


FIG. 4. Separation of AMP analogs on HLADH-silica. The binding-site concentration in the column was 200 μM . Sample concentrations: 40–100 μM . Flow rate: 0.5 ml/min.

be inaccessible to a macromolecular solute/enzyme and that the effective concentration will be difficult to assess. However, in the present case the situation is more favorable. The small solute molecules would have unhindered access to the macromolecular enzyme and, provided that the enzyme is properly oriented on the silica surface, the interaction with the two binding sites on the enzyme should also be feasible. The effective ligand concentration could thus be expected to be approximately the same as the nominal one, an assumption supported by the equilibrium-binding study referred to in Table 2. A way of circumventing any difficulty in correctly determining the effective concentration of immobilized ligand was practiced by Brodelius and Mosbach (24), who constructed a standard curve of chromatographic data from well-characterized substances and then used the curve for characterizing unknown complexes.

A number of adenine nucleosides and nucleotides were chromatographed on HLADH-silica and k' values and dissociation constants were calculated (Table 3). All data in the table refer to a flow rate of 1 ml/min. It was shown that k' varied less than 10% in the interval 0.1–3 ml/min. The correlation between calculated and available literature values are in most cases satisfactory. The calculated values are generally higher, which may be explained

TABLE 3

CHROMATOGRAPHIC DATA FOR ADENINE NUCLEOSIDES AND ADENINE NUCLEOTIDES

Compound	Spacer composition	k' value	K_d (μ M)	
			Calculated	Literature
Adenosine derivatives				
Adenosine		0.25	1150	3700
N^6 -(6-Aminohexyl)-ado	$H_2N(CH_2)_6-$	1.56	185	
N^6 -(N -(6-Aminohexyl)-carbamoylmethyl)-ado	$H_2N(CH_2)_6NHCOCH_2-$	0.40	720	
AMP derivatives				
AMP		2.15	134	70
N^6 -(6-aminoethyl)-AMP	$H_2N(CH_2)_6-$	5.6	51	32
N^6 -(2-aminoethyl)-AMP	$H_2N(CH_2)_2-$	0.28	1030	
N^6 -carboxymethyl-AMP	$HOOCCH_2-$	2.7	107	
ADP		0.19	1500	390
ATP		0.06	5000	
ADP-ribose		7.5	38	18
NAD derivatives				
NAD		2.4	120	96
N^6 -(N -(6-aminoethyl)-carbamoylmethyl)-NAD	$H_2N(CH_2)_6NHCOCH_2-$	1.56	185	
N^6 -(N -(2-aminoethyl)-carbamoylmethyl)-NAD	$H_2N(CH_2)_2NHCOCH_2-$	0.40	720	
NADP derivatives				
N^6 -(N -(6-aminoethyl)-carbamoylmethyl)-NADP	$H_2N(CH_2)_6NHCOCH_2-$	0.05	6000	

Note. HLADH-Silica (1000 Å, 10 μ M) with an enzyme content of 21 mg/g silica, giving an average subunit concentration of 288 μ M in the column, was used. Literature values were obtained from Ref. (24) (N^6 -(6-aminoethyl)-AMP), Ref. (25) (Adenosine, AMP, ADP-ribose), Ref. (26) (ADP), and Ref. (27) (NAD).

by the fact that the chromatography was carried out with high-ionic-strength phosphate buffer ($I = 0.5$ M), whereas literature data refers mainly to low-ionic-strength phosphate buffers ($I = 0.1$ M). Phosphate buffer of high ionic strength is known to increase K_d for free HLADH (16). Table 3 indicates that the K_d 's of weakly retained substances agree poorly with literature data, probably because the precision in k' determination is less satisfactory for these substances.

The AMP derivatives show interesting differences in K_d , differences that may be rationalized by knowledge obtained from X-ray crystallographic studies of HLADH-ADP-ribose complexes, assuming analogous binding

in all cases. The adenine ring in NAD and ADP-ribose is sandwiched between isoleucine residues 224 and 269 (28,29). The N^6 amino group points away from the enzyme into the solution but is partly shielded by the side chain of arginine 271. This latter residue is close enough to attract the negatively charged carboxymethyl group of N^6 -carboxymethyl-AMP and to repel the positively charged amino group of N^6 -aminoethyl-AMP. This would explain why the carboxymethyl derivatives have a smaller K_d value and why the aminoethyl derivatives have a larger K_d value than native AMP.

In the case of N^6 -(6-aminoethyl) derivatives of adenosine and AMP, it could be argued

that the spacer positions the positively charged group away from the arginine 271 residue and allows it to be attracted by the carboxyl group of Asp 273. Alternatively, the hexyl spacer may interact with the hydrophobic milieu around the adenine-binding site. Both explanations could account for the lower K_d values calculated for the aminohexyl derivatives compared to those of the parent compounds.

Interestingly, the calculated K_d values for 6-aminohexyl carbamoylmethyl derivatives are higher than those of the parent compounds. This is in accordance with observations made by Andersson in systems comprised of Sepharose-bound NADH analog and free enzyme (30). The reason for the lower affinity is possibly that a less flexible and less hydrophobic portion has been inserted in the spacer between the adenine and the aminohexyl entity, preventing an affinity-enhancing contact with the enzyme.

ADP and ATP bound poorly to HLADH-silica, illustrating the difficulty for the pyrophosphate-binding site (29) of accepting more than two negative charges.

Elution with counter ligands. Table 3 does not include NADH due to its overly tight binding to alcohol dehydrogenase. The K_d for NADH equals $0.6 \mu\text{M}$ (16), which can be expected to give an impractically high k' value with the column used ($k' = 500$). However, the k' value with a given column can be influenced by high concentrations of salts (16), elevated temperature (31), and addition of organic solvents (32).

A more specific way of reducing k' is to include a competitive ligand in the eluant. The dependence of k' on the concentration and dissociation constant of the competing ligand (C) has been worked out for the enzyme in solution (20,21). The analogous expression in our case will be

$$k' = \frac{[\text{ADH}]}{K_d(\text{NADH})} \cdot \frac{K_d(\text{C})}{K_d(\text{C}) + [\text{C}]} \quad [6]$$

The validity of the expression was checked with NADH, using AMP and NAD as counter

ligands. From the resulting elution profiles the K_d for NADH was calculated to be $0.63 \mu\text{M}$ (using AMP as counter ligand) and $1.0 \mu\text{M}$ (with NAD as counter ligand). The values are in excellent agreement with those obtained with equilibrium measurements using the same ionic strength (Table 3) and with literature values (16). The option of using counter ligands to modify the retention thus seems to conveniently extend the useful range of a given affinity column either when it is used for analytical separations or for determination of dissociation constants.

pH effects on k' . The binding of coenzyme and coenzyme fragments to liver alcohol dehydrogenase is pH dependent (25,33). This was easily demonstrated for the silica-bound enzyme as well. In Table 4 the pH effects on k' for ADP-ribose and NAD are presented and the corresponding dissociation constants calculated. As can be seen, the k' for NAD is strongly influenced by the pH. This knowledge could be utilized to separate AMP from NAD, both substances having about the same k' at pH 7.5 (Table 3). Thus at pH 7.0 NAD eluted before AMP and at pH 8.0 it eluted after AMP. The pH effect on the K_d for ADP-ribose and NAD seems to be the same for immobilized

TABLE 4
THE pH INFLUENCE ON k' AND K_d WITH
HLADH-SILICA

Compound	pH	k'	$K_d (\mu\text{M})$	
			Calculated	Literature
NAD ⁺	7.0	0.45	266	133
	7.5	0.77	156	96
	8.0	1.93	62	48
	8.3	2.68	45	30
ADP-ribose	7.0	3.1	38	16
	7.5	3.0	40	18
	8.0	2.9	42	21
	8.3	2.5	48	25

Note. The binding-site concentration in the column was $120 \mu\text{M}$. Sample concentration: $100 \mu\text{M}$. Flow rate: 1 ml/min. The literature values were obtained from Ref. (27) (NAD) and from Ref. (25) (ADP-ribose).

HLADH as for the free enzyme, the differences in absolute values between our data and literature values probably being caused by the higher concentration of phosphate buffer that we used (16).

Chromatography of dyes on HLADH-silica.

Triazine dyes are finding increasing use as general ligands in affinity chromatography (34). We applied three different triazine dyes (technical grade) to an HLADH-silica column, after first having treated them in alkali to remove the reactive chlorine groups (Fig. 5). Procion Yellow MX-8G did not bind, whereas Procion Red MX-5B was retarded and Cibacron Blue F3G-A did not elute. Procion Red was resolved into at least five components, the major one having a k' of about 2. No change in the elution behavior of any of the dyes occurred when 2 mM AMP, 2 mM NAD, or 2 mM NADH was included in the eluant. Since NADH is a very strong competitive eluant ($K_d \approx 0.6 \mu\text{M}$) this clearly indicates that at least Procion Red was not retarded due to interaction with the coenzyme-binding site. The possibility remains that Cibacron Blue was adsorbed to the column due

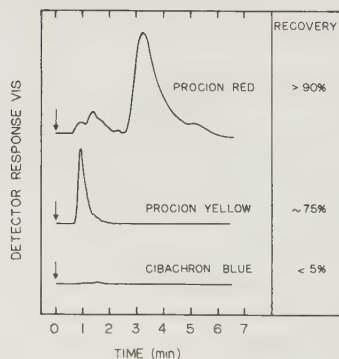


FIG. 5. Chromatography of triazine dyes on HLADH-silica. The binding-site concentration in the column was $37 \mu\text{M}$. Prior to injection on the column, the dyes were deactivated by treatment with 0.1 M NaOH for 16 h at room temperature and then diluted with the irrigating buffer to $100 \mu\text{M}$. Procion Red was detected at 540 nm, Procion Yellow at 450 nm, and Cibacron Blue at 620 nm. Flow rate: 1 ml/min .

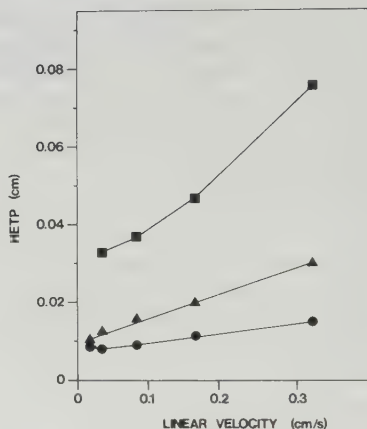


FIG. 6. HETP as a function of the linear velocity on HLADH-silica. The k' values for adenosine, AMP, and ADP-ribose were 0.12, 1.2, and 3.3, respectively. Sample concentration: $20 \mu\text{M}$.

to a very strong interaction with the coenzyme-binding site. None of the dyes was retarded on a diol-silica reference column.

Plate heights and determination of rate constants. The resolution was, as expected, dependent on the flow rate. As seen in Fig. 6, the plate heights (19) were quite small at low flow rates, down to 10^{-2} cm for adenosine and AMP, which is comparable with values in conventional HPLC. Kasche *et al.* (3) obtained plate heights of about 1 cm when they chromatographed chymotrypsin on STI columns. The marked difference is probably caused by the slow diffusion of the high-molecular-weight chymotrypsin, compared to the small coenzymes and coenzyme fragments used here. Furthermore, the low dissociation rate of the STI-chymotrypsin complex will increase the plate height.

The plate height (HETP) for NAD was found to be pH dependent, increasing from 0.11 cm at pH 7.5 to 0.19 cm at pH 8 (1 ml/min). The plate heights for AMP and ADP-ribose were, on the other hand, not significantly affected. This increase for HETP of NAD is most probably a reflection of a decreased dissociation rate for NAD at pH 8

when compared to pH 7.5, as is also reported in the literature (27,33). At least a qualitative relationship thus exists between peak width/plate height and the rate constants for the involved binary complexes. Several quantitative relationships between chromatographically obtained data and rate constants have recently been proposed by Denizot and Delaage (35), Horvath and Lin (36), and Hethcote and DeLisi (37). However, when our data were combined with the suggested relationships a considerable difference between calculated values and literature values was obtained. Using a method by Horvath and Lin (36) as modified by Kasche *et al.* (3), the dissociation rate constants (k_{off}) for AMP, ADP-ribose, and NAD were calculated as 5.4, 1.4, and 0.44 s^{-1} , respectively. The literature value for NAD is two orders of magnitude higher (40 s^{-1} (27)). Using the method of Denizot and Delaage gave even lower rate constants.

The possibility of determining rate constants of biological complexes by chromatographic means is an interesting prospect due to the simplicity of the method. However, the large discrepancy between calculated constants and those reported from other determinations calls for a careful analysis of the prerequisites for making the method applicable. One explanation for the poor agreement is the possibility that the intraparticle diffusion resistance is not adequately compensated for.

Further, the rate constants related to silica-bound and free enzyme are not necessarily identical even if the bound enzyme is physically unharmed by the immobilization and even if the involved equilibrium constants are identical. For example, the silica-bound molecules may contain a small and otherwise hard to detect subpopulation of slowly interacting sites (e.g., caused by steric shielding). Such a subpopulation can be envisaged to govern the whole separation process provided the introduced sample is small enough. Although, as discussed in connection with Table 2, Scatchard plots and activity measurements indicated a homogeneous population of binding sites, a clear indication of inhomogeneity was

observed when making *in situ* active-site titrations of column-packed HLADH-silica (Fig. 7). In this experiment NADH + isobutyramide were continuously pumped through HLADH-silica columns and the absorbance of the effluent recorded. The breakthrough was very distinct in case of the low-substituted silica (Fig. 7B), but less so in case of the more densely loaded silica (Fig. 7C). In the latter case the curve is less steep, indicating the presence of a slow binding process.

Stability of HLADH-silica. Although no experiments were designed for the sole purpose of measuring the stability of HLADH-silica, a function-related estimate was obtained anyhow, simply by following the decrease of k' with time for chromatographed substances. The stability was found to vary in a not easily predictable way. For example, a set of HLADH columns was operated continuously for 3 days at room temperature with fully retained performance. In another case, under apparently the same conditions (but using another HPLC set up), a 50% decrease of retention was observed. The k' values decreased proportionally for all the tested substances.

CONCLUSIONS

The tresyl chloride-activation method appears to be ideally suited for the coupling of

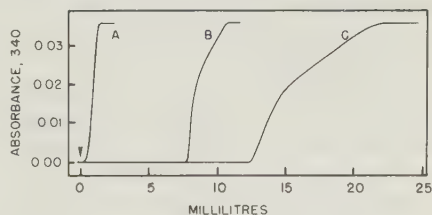


Fig. 7. *In situ* active-site titration of HLADH-silica. Mobile phase: 6 μM NADH, 0.15 M isobutyramide, 1 μM $ZnSO_4$, 0.25 M sodium phosphate buffer, pH 7.5. Flow rate: 0.3 ml/min. The profiles were obtained by directing the flow through a bypass loop (A), through a low-activated/low-substituted HLADH-silica column (B), and through a high-activated/high-substituted HLADH-silica column (C).

sensitive molecules such as enzymes to silica. Liver alcohol dehydrogenase was thus coupled in yields close to 100% with preserved integrity as judged from active-site titrations, activity measurements, and binding studies.

The HLADH-silica had the capacity of resolving a number of nucleosides, nucleotides, as well as various dyes. The potential of the adsorbent was best demonstrated during the separation of closely related adenine nucleotide analogs, differing with regard to substituents in the N⁶ position. The separation pattern could be rationalized by the interaction between the N⁶ substituent and certain specified amino acid residues on the enzyme surface. The HPLAC technique thus appears well suited for the rapid screening of interaction patterns between biomolecules and possibly also for deduction of surface composition and macromolecular architecture.

Quantitative treatment of chromatographic data provided dissociation constants for the complexes involved. The values obtained were in good agreement with literature data for the corresponding soluble complexes, emphasizing the point that a mild immobilization method will preserve the intrinsic molecular properties. Determination of dissociation and association rate constants with HPLAC technique was, on the other hand, unsuccessful. To fully realize this very interesting prospect, the system must be carefully scrutinized in order to minimize or correctly assess all other contributions to band spreading other than those which are due to the association/dissociation processes.

The influence of temperature on k' was not investigated, but it should be possible to rapidly determine the enthalpic and entropic contributions in the binding process for a number of compounds from such a study.

HLADH-silica should also be a useful adsorbent for a number of substrates, inhibitors, and other compounds by utilizing ternary-complex formation during the separation process. This approach has not yet been pursued, but in line with this was the unexpected observation that the presence of dithioerythritol

in the eluant markedly influenced the retention of NAD.

Finally, it should be pointed out that affinity chromatography in the "high-performance" mode with immobilized macromolecules should obviously be applicable to enzymes/binding molecules other than alcohol dehydrogenase.

ACKNOWLEDGMENTS

The financial support from The Biotechnology Research Foundation (Sweden) and The Swedish Natural Research Council is gratefully acknowledged.

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